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REVIEW ARTICLE

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Mechanisms and roles of mitophagy in neurodegenerative diseases

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Abstract

Mitochondria are double-membrane-encircled organelles existing in most eukaryotic cells and playing important roles in energy production, metabolism, Ca^{2+} buffering, and cell signaling. Mitophagy is the selective degradation of mitochondria by autophagy. Mitophagy can effectively remove damaged or stressed mitochondria, which is essential for cellular health. Thanks to the implementation of genetics, cell biology, and proteomics approaches, we are beginning to understand the mechanisms of mitophagy, including the roles of ubiquitin-dependent and receptor-dependent signals on damaged mitochondria in triggering mitophagy. Mitochondrial dysfunction and defective mitophagy have been broadly associated with neurodegenerative diseases. This review is aimed at summarizing the mechanisms of mitophagy in higher organisms and the roles of mitophagy in the pathogenesis of neurodegenerative diseases. Although many studies have been devoted to elucidating the mitophagy process, a deeper understanding of the mechanisms leading to mitophagy defects in neurodegenerative diseases is required for the development of new therapeutic interventions, taking into account the multifactorial nature of diseases and the phenotypic heterogeneity of patients.

KEYWORDS
LC3 adapters, mitochondria, mitophagy, mitophagy receptors, neurodegenerative diseases, Parkin, PINK1, ubiquitin

1 | INTRODUCTION

As key organelles widely distributed in most eukaryotic cells, mitochondria are indispensable for diverse cellular processes, from generating adenosine triphosphate (ATP), maintaining calcium homeostasis, synthesizing key metabolites, producing endogenous reactive oxygen species (ROS), to regulating necrosis, apoptosis, and autophagy.[1-3] Therefore, appropriate regulation of mitochondrial health is of crucial importance to various physiological and pathological processes. In yeast, deletion of the *Mdm38* gene product, an inner mitochondrial membrane (IMM) protein with K^+/H^+ exchanger activity, can reduce the content of respiratory chain complex, induce mitochondrial morphological changes, break the homeostasis of mitochondrial K^+, and lead to mitophagy.[4] Mitochondria are removed by the process of autophagy when mammalian reticulocytes mature into erythrocytes.[5] In fertilized *Caenorhabditis elegans* oocytes, the sperm-derived mitochondria would be removed by mitophagy.[6,7] These results suggest that maintaining mitochondrial homeostasis by mitophagy plays an important role in the differentiation and development of eukaryotes.

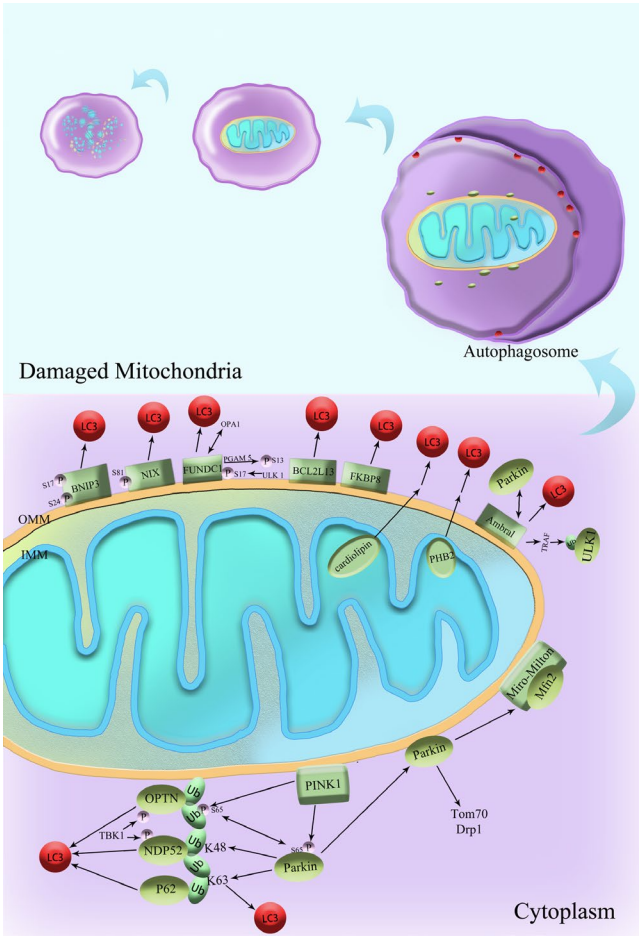

FIGURE 1 Mechanisms of Mitophagy in mammals. Some OMM proteins, such as BNIP3, NIX, and FUNDC1, possess the LC3-interacting regions (LIRs) and can interact with LC3. Thus, the interaction between the LIRs of mitophagy receptors and LC3 is considered a crucial link in selecting mitochondria as the cargo. Moreover, reversible protein phosphorylation is suggested to effectively regulate the receptor-mediated mitophagy. PINK1 and Parkin synergistically regulate mitophagy in mammals in the same pathway. In the damaged mitochondria, mitochondrial membrane potential is lost and PINK1 accumulates on the membrane. PINK1 would recruit Parkin to the damaged mitochondria and phosphorylate Parkin. Parkin can stimulate the ubiquitination of its substrates on OMM via K48 or K63 linkage, followed by protein quality control and subsequent mitochondrial quality control. Many autophagy adaptor proteins are involved in this process, such as p62, NBR1, OPTN, NDP52, and TAX1BP1. Ambra1, activating molecule in beclin 1-regulated autophagy; BCL2L13, BCL2-like 13; BNIP3, Bcl2/adenovirus E1B 19-kDa protein-interacting protein 3; Drp1, dynamin-related protein 1; FKBP8, FK506-binding protein 8; FUNDC1, FUN14 domain-containing protein 1; LC3, microtubule-associated protein 1 light chain 3; Mfn 2, mitofusin 2; NDP52 (CALCOCO2), calcium-binding and coiled-coil domain 2; NIX (BNIP3L), BCL2/adenovirus E1B 19-kDa interacting protein 3-like; OPTN, optineurin; P, phosphate; p62 (SQSTM1), sequestosome 1; PGAM5, phosphoglycerate mutase family member 5; PHB2, Prohibitin 2; PINK1, PTEN-induced putative kinase 1; TOM70, translocase of outer mitochondrial membrane 70; TRAF, tumor necrosis factor receptor-associated factor; Ub, ubiquitin; ULK1, UNC51-like kinase 1

Mitochondrial dysfunction has been implicated in numerous neurodegenerative diseases including Parkinson's disease (PD). PD is characterized by the degeneration of dopaminergic neurons in the midbrain. Mitochondrial dysregulation can lead to PD.[8-11] Mutations in *PINK1* and *Parkin* genes are associated with autosomal recessive early-onset PD.[12,13] *PINK1* and *Parkin* genes exert synergistic effect on mitochondrial maintenance-related functions, such as mitochondrial motility, proteasomal degradation of mitochondrial proteins, and mitophagy. Additionally, Parkin also participates in removing mitochondria during the progression of Alzheimer's disease (AD), and the overexpression of Parkin can alleviate symptoms of AD.[14] Supporting a role of mitophagy in AD, amyloid beta-derived diffusible ligands (ADDLs) can induce the fragmentation of mitochondria and mitophagy.[15-17] Mitophagy is also altered in HD, and the mutant huntingtin may induce selective autophagy.[18] In a cell culture model of HD, excessive mitochondrial fission partially mediates cytotoxicity.[19] Mitochondrial dysfunction has also been observed in ALS, and reduced targeting of ubiquitinated mitochondria to autophagosomes may contribute to the pathogenesis of ALS.[20] Mitophagy thus plays a multifaced role in neurodegenerative diseases.

2 | SELECTIVE AND NONSELECTIVE AUTOPHAGY

Under certain conditions, organelles, together with bits of cytoplasm, will be sequestered and degraded by lysosomes for hydrolytic digestion in a process termed autophagy.[21] Generally, autophagy can be classified into nonselective autophagy and selective autophagy, with the former being primarily a starvation response, while the latter eliminating damaged organelles and remodeling cells to adapt to environmental changes.[22] Defects in selective autophagy can result in a range of human pathophysiologies, including certain types of neurodegenerative diseases. Macroautophagy is an evolutionarily conserved process that allows cells to degrade and recycle cytoplasm. Whereas nonselective macroautophagy can randomly engulf a portion of cytoplasm into autophagosome and subsequently transfer it to the vacuole or lysosome for degradation, selective macroautophagy can specifically identify and degrade specific substances, such as protein complexes, organelles, or invading microorganisms.[23] The morphological hallmark of macroautophagy is the formation of an initial sequestering compartment, the phagophore, which can expand into the double-membrane autophagosome, and the initial sequestration occurs in a compartment separate from the degraded organelle. Typically, selective macroautophagy can be classified into mitophagy, pexophagy, reticulophagy, ribophagy, etc. In higher eukaryotes, selective autophagy also includes chaperone-mediated autophagy (CMA), as well as two similar processes, namely endosomal microautophagy (e-MI) and chaperone-assisted selective autophagy (CASA).[24] Nonetheless, how a substrate is targeted for sequestration and segregated from other cellular parts remains one of the major issues in this research field.

3 | MITOPHAGY: SELECTIVE AUTOPHAGY OF MITOCHONDRIA

The by-products of mitochondrial metabolism can induce DNA damage or genetic mutation.[25] Therefore, mitochondrial health is found to play a vital role in cellular health. Mitophagy, a selective autophagy process, is essential for mitochondrial health.[26] ATP is the product of oxidative phosphorylation, which can result in ROS production within mitochondria. Excessive mitochondrial ROS can cause cytotoxicity and lead to cell death under some conditions. Mitochondria are particularly vulnerable to ROS damage due to the "naked" nature of their DNA and limited antioxidant capacity inside mitochondria. If not properly repaired or cleared from cells, the unhealthy mitochondria will cause further production of ROS and release of proapoptotic proteins into the cytosol, finally leading to a high risk of cell death.[27]

Mitophagy, first discovered by Lewis and Lewis[28] in cells, is a kind of selective autophagy that handles dysfunctional or damaged mitochondria, which tend to accumulate following mitochondrial damage or stress. Ashford and Porter[29] employed electron microscopes to observe the mitochondrial debris in 1962, and it was suggested that functional alterations of mitochondria would trigger their autophagy.[30] The term "mitophagy" came into use in 1998.[31]

Mitophagy is required to remove the unhealthy mitochondria timely and to maintain steady mitochondrial turnover, as well as cellular development and differentiation.[32] Notably, selective degradation of the surplus or dysfunctional mitochondria by autophagy has been observed in organisms ranging from yeast to mammals. In yeast, mitophagy is mediated by Atg32, which is related to NIX and its regulator Bcl2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) in mammals. Numerous studies in metazoans suggest that mitophagy is mostly regulated by PINK1 and Parkin, which are not present in yeast, suggesting species-specific difference in mitophagy regulation. Mitophagy is one of the most extensively investigated types of "organelle autophagy," which can be partially ascribed to the connection between mitophagy and disease.

4 | MITOPHAGY IN MAMMALS

In mammalian cells, mitophagy is activated by two distinct pathways, one is dependent on receptors while the other one relies on ubiquitin (Figure 1). Although both types of mitophagy, namely the receptor-mediated and PINK1/Parkin-mediated mitophagy, have been well studied recently, it remains unknown about how these two types of mitophagy differ in expression among different tissues or in output of mitochondrial degradation.[33] In mammals, both NIX (also known as BNIP3L) and SQSTM1/p62 have been implicated to function as the receptors linking mitochondria with the autophagy mechanism in different cell types. During erythrocyte maturation, NIX is essential for mitochondrial clearance, where mitophagy plays a developmental role. Autophagosome can recognize target mitochondria through LC3 adapters (in ubiquitin-dependent and ubiquitin-independent manners) and the direct interaction of LC3 with its receptors.[34]

4.1 | Mitophagy receptors in mammals

The molecular mechanisms of mitophagy in mammals appear to be quite distinct from that in yeast. The mammalian homolog of yeast Atg32 has not been identified. Some functional counterparts of Atg32, such as BNIP3, NIX, and FUN14 domain-containing protein 1 (FUNDC1), have been suggested to serve as the mitophagy receptors in mammals.[35] All these receptors can be recruited to the OMM, and they possess the LC3-interacting regions (LIRs) and can interact with LC3, a mammalian homolog of Atg8. Thus, the interaction between the LIRs of mitophagy receptors and LC3 is recognized as a crucial link in selecting mitochondria as the cargo. Moreover, reversible protein phosphorylation is suggested to effectively regulate the receptor-mediated mitophagy. For example, under normoxic conditions, Src and CK2 can phosphorylate FUNDC1 and suppress mitophagy; however, under hypoxia conditions, phosphoglycerate mutase 5 (PGAM5) can dephosphorylate FUNDC1 and thereby effectively promote mitophagy.[36,37]

4.1.1 | BNIP3 and NIX/BNIP3L in mammalian mitophagy

BNIP3 and its homologous protein BNIP3L/NIX are BH3-only proteins, belonging to the BCL2 family. BNIP3 and NIX function in cell death and work together with the LC3 family proteins to remove mitochondria,[38] with NIX being implicated in reticulocyte maturation.[39] Atg7 and ULK1 are also involved in mitochondrial removal in reticulocytes. Moreover, nonautophagic mechanisms may also promote mitochondrial removal during reticulocyte maturation.[40] NIX/BNIP3L and BNIP3, which are regulated by hypoxia-inducible factor (HIF) or forkhead homeobox type O (FOXO), also participate in the hypoxia-induced mitophagy.[41]

While the upregulation of NIX or BNIP3 can enhance their activities in mitophagy, the interplay between BNIP3 and LC3 may also act at the level of phosphorylation of BNIP3. When Ser17 and Ser24 adjacent to the LIRs of BNIP3 are phosphorylated, the interplay between BNIP3 and LC3 is enhanced, suggesting a possible role of kinases or phosphatases in regulating mitophagy. Additionally, the interaction between NIX and a small GTPase Rheb is shown to initiate mitophagy.[42]

4.1.2 | FUNDC1-mediated mitophagy

FUNDC1 is an OMM protein associated with the hypoxia-induced mitophagy. Under hypoxia condition, the FUNDC1 mRNA and protein levels are dramatically downregulated, which may be attributable to mitophagy. No conserved HIF-1 recognition site is found in the promoter region of FUNDC1.[43] FUNDC1 can effectively activate the autophagy mechanism and promote mitophagy. The FUNDC1-dependent mitophagy may also contain

a Beclin1-independent component.[44] Mutations in the Y(18) and L(21) conserved sites of the FUNDC1 LIRs, together with the phosphorylation of Tyr18 and Ser13 can effectively inhibit the interplay with LC3.[45]

FUNDC1 is phosphorylated at Ser17 by ULK1 and dephosphorylated at Ser13 by PGAM5 under hypoxia condition. BCL2L1/Bcl-X_L can not only inhibit the PGAM5 phosphatase activity, but also interact with PGAM5 and block the dephosphorylation of FUNDC1.[46] Under hypoxia condition, ULK1-mediated phosphorylation at Ser17 can promote the interaction between FUNDC1 and LC3.[47] Additionally, the mitochondrial E3 ligase MARCH5 can regulate hypoxia-induced mitophagy through ubiquitinating and degrading FUNDC1.[48] The receptor-interacting serine/threonine-protein kinase 3 (Ripk3) can suppress FUNDC1-mediated mitophagy and promote mitochondrial apoptosis in cardiac ischemia/reperfusion injury.[49] Unexpectedly, it is also suggested that knockdown or overexpression of FUNDC1 has insignificant influence on starvation- or hypoxia-induced mitophagy. The cause of such divergent results remains to be fully clarified.

In mammalian cells, FUNDC1 can recruit LC3 through its LIR motif, thereby activating mitophagy; it can also interact with DNM1L/DRP1 and OPA1, regulating mitochondrial fission or fusion, and mediating mitophagy. OPA1 can interact with FUNDC1 through its Lys70 (K70) residue, and mutation of K70 to Ala (A) will eliminate the interaction between OPA1 and FUNDC1, and promote mitochondrial fission and mitophagy.[50] Therefore, FUNDC1 can coordinate the dynamics and quality control of mitochondria.

4.1.3 | BCL2L13 and FKBP8

Bcl2-like 13 (BCL2L13), which is located on the OMM and can bind to LC3 via the LIR motif, contains one transmembrane region and one LIR on its N-terminal facing the cytoplasm. BCL2L13 has been identified as one of the functional counterparts of Atg32, since exogenous BCL2L13 expression can partially rescue a mitophagy defect in the atg32Δ yeast. Similar to the case of Atg32, the phosphorylation of Ser272 on BCL2L13 can also stimulate the binding of BCL2L13 to LC3.[51,52]

FK506-binding protein 8 (FKBP8) is an LC3-interacting protein located on the OMM, which can effectively promote mitophagy in a Parkin-independent manner.[53] In particular, FKBP8 migrates from mitochondria into ER following treatment with CCCP, a chemical ionophore. The FKBP8 N412K mutant that cannot translocate to ER is defective in suppressing apoptosis during mitophagy, suggesting that not all mitochondrial proteins are degraded during mitophagy, and that the subcellular localization of FKBP8 can regulate cell survival during mitophagy.[54]

4.1.4 | PHB2 and cardiolipin

Although prohibitin 2 (PHB2) is located in the IMM, Parkin-mediated degradation of OMM proteins can trigger the rupture of OMM, exposing PHB2 to LC3 and finally inducing mitophagy. In *C elegans*, PHB2 plays a vital role in the removal of damaged mitochondria.[55,56]

Cardiolipin is a kind of membrane lipid existing in the IMM, which can also serve as a receptor of LC3 in mitophagy. Cardiolipin can be transferred from IMM to OMM during mitochondrial depolarization to induce mitophagy. Cardiolipin and Parkin can independently respond to CCCP treatment and regulate mitophagy at different levels of mitochondrial depolarization.[57,58]

Externalized cardiolipin can directly interact with the N-terminal helix of LC3, which is specific to the LC3 subtype. In rotenone-treated cells, cardiolipin can directly interact with gamma-aminobutyric acid receptor-associated protein (GABARAP), one of the LC3 family members, but it would not recruit GABARAP to mitochondria. Thus, cardiolipin can interact with GABARAP during different autophagic processes. These findings indicate that the IMM components also participate in mitophagy.[59]

4.1.5 | Ambra1

Ambra1, a Beclin 1 interactor, is another mitophagy receptor. After autophagy induction, Ambra1 can gradually transfer from the cytoskeleton to ER and regulate autophagosome nucleation.[60] The LIR region in Ambra1 can directly bind to LC3 during mitophagy induction. Targeting Ambra1 on mitochondria can promote mitophagy in either Parkin-dependent or Parkin-independent pathways. In *Parkin*-deficient cells, Ambra1 is subject to ubiquitination when Ambra1 is targeted to mitochondria.[61,62] Ambra1 also promotes ubiquitination of ULK1 through the E3 ligase tumor necrosis factor receptor-associated factor 6 (TRAF6), indicating that Ambra1 is probably the adaptor for E3 ligases.[63]

It is demonstrated that Parkin can interact with Ambra1, and prolonged mitochondrial depolarization will further enhance their interaction. Moreover, Ambra1 can be recruited to depolarized mitochondria and eventually promote mitophagy, and Parkin translocation can trigger mitophagy through the activation of Ambra1 and the ubiquitination of OMM proteins.[64]

4.2 | Ubiquitin-mediated interaction of LC3 adapters

4.2.1 | PINK1/Parkin-mediated mitophagy

PINK1 and Parkin mutations are the most common pathogenic factors of recessive familial PD. PINK1 is a mitochondria-targeted serine/threonine kinase, while Parkin is a cytoplasmic E3 ubiquitin ligase. PINK1 and Parkin function in the same pathway and to mediate mitophagy in metazoans, with PINK1 acting upstream of Parkin.[65-69] PINK1 is also regarded as a mitochondrial stress sensor whose function depends on the mitochondrial membrane potential.[70-72]

The PINK1-Parkin pathway may be responsible for regulating the heterogeneity between the healthy and damaged mitochondria and thus mitochondrial homeostasis in cells. Parkin and PINK1 can promote mitochondrial health through several mitochondrial quality control mechanisms, including the turnover of OMM proteins by the proteasome, the generation of mitochondrial-derived vesicles,

and organellar degradation through mitophagy. Under healthy mitochondrial condition, PINK1 is maintained at a low level through complex processing and degradation. But PINK1 is stabilized on OMM with decreased mitochondrial membrane potential, where PINK1 can recruit Parkin to damaged mitochondria.[73] Thereafter, PINK1 can phosphorylate Parkin at Ser65 and stimulate Parkin's E3 ligase activity[74]; PINK1 also phosphorylates ubiquitin at Ser65, which would then activate Parkin upon binding.[75-77] It is thought that the ubiquitination of OMM proteins by Parkin can initiate the mitophagy process. Parkin can stimulate the attachment of the ubiquitin chain to its substrates through K48 and K63 linkages. Normally, protein degradation can be initiated by K48-linked ubiquitination, which can initiate passive mitochondrial degradation. The autophagy-associated adaptors LC3/GABARAP are generally recruited by K63-linked ubiquitination, which is involved in mitophagy.[78]

After being recruited to the mitochondria, Parkin directs the ubiquitination of various OMM proteins, which can mediate mitochondrial sequestration through interaction with the adaptor proteins on the separation membrane. Several substrates of Parkin have been identified, including mitofusin (Mfn), TOM70, Miro, and Drp1, suggesting that Parkin plays multifaceted roles in the dynamics and biogenesis of mitochondria. p62 is suggested to be recruited to mitochondria along with Parkin; however, p62 is reported to be unnecessary for removing the damaged mitochondria, challenging the role of p62 in the PINK1/Parkin-mediated mitophagy.[79-81]

The mitochondrial fusion GTPase Mfn2 is also a Parkin substrate. Mfn2 can interact with the Miro-Milton complex on mitochondria and is therefore also related to the transport of mitochondria.[82] Mfn2 can be phosphorylated by PINK1 and subsequently ubiquitinated by Parkin. As a Parkin receptor, Mfn2 can induce the migration of Parkin to the damaged mitochondria, and the PINK1/Parkin-mediated Mfn2 ubiquitination may induce mitophagy.[83,84] However, the ongoing mitophagy in Mfn (−/−) cells suggests that Mfn is not absolutely required for Parkin-mediated mitophagy.[85]

4.2.2 | LC3 adapters: p62, NBR1, OPTN, NDP52, and TAX1BP1

The autophagy adaptor proteins, including SQSTM1 (also called p62), NBR1, optineurin (OPTN), NDP52 (also called CALCOCO2), and TAX1BP1, possess the ubiquitin-binding domains, which can interact with the ubiquitinated mitochondrial proteins, and the LIR motifs, which can recruit a separation membrane through interacting with LC3, in the selective autophagic degradation of mitochondria.[86-88] Among them, OPTN is one of the most studied adapters for the recruitment of phagophore to the mitochondria mediated by the PINK1/Parkin pathway.[89] OPTN can be recruited to damaged mitochondria through binding with ubiquitinated OMM proteins, thereby inducing mitochondrial isolation by autophagosome through its interaction with LC3.[90] TBK1 is activated after recruitment by OPTN, which can then promote mitophagy.[91,92] Though TBK1 can phosphorylate various adapters, only OPTN and NDP52 have been regarded as its primary substrates.[93,94] PINK1 has also been shown to play a role in the mitophagy pathway in a Parkin-independent manner. PINK1 recruits OPTN and NDP52 onto the mitochondria, as well as the subsequent recruitment of autophagy initiation factors, including ULK1 and double FYVE-containing protein 1 (DFCP1). Interestingly, overexpression of synphilin-1, an alpha synuclein-interacting protein, can induce PINK1 accumulation. Later, the PINK1-synphilin-1 complex can recruit an E3 ubiquitin ligase (SIAH) to enhance mitochondrial ubiquitination, indicating that E3 ligase other than Parkin can target mitochondria for degradation in distinct ways.[95]

5 | MITOPHAGY AND NEURODEGENERATIVE DISEASES

Mitophagy often takes place under baseline conditions; however, it can also be promoted under specific physiological conditions. For example, NIX can remove mitochondria from the mature erythrocytes during development, and Parkin and the mitochondrial E3 ubiquitin protein ligase 1 (MUL1) are necessary for the degradation of paternal mitochondria after fertilization in mice.[96] The differentiation state of stem cells can be affected by the PINK1-dependent mitophagy.[97] On the one hand, mitophagy contributes to metabolic changes during the differentiation and the transition from beige to white adipocytes.[98] On the other hand, mitophagy also exerts great roles in activating the NOD-like receptor protein 3 (NLRP3) inflammasome, and the FUNDC1-dependent mitophagy can downregulate platelet activation following acute ischemia/reperfusion injury.[99,100] As reviewed below, defective mitophagy is frequently observed in neurodegenerative diseases (Table 1).

5.1 | Mitophagy and PD

Parkinson's disease is characterized by the selective loss of dopaminergic neurons in the pars compacta of the substantia nigra and features clinical phenotypes of rigidity, resting tremor, postural instability, and bradykinesia. Mitochondrial dysfunction has been consistently recognized as an important contributor to the pathogenesis of PD. Major features of mitochondrial dysfunction in PD include ROS overproduction, ATP depletion, mitochondrial DNA depletion, and caspase release. Mitochondrial toxins rotenone and MPTP can impair the mitochondrial function through inhibiting the activity of complex I, whereas suppression of complex I could result in abnormal OPA1 oxidation, which would subsequently lead to abnormality in mitochondrial structure and neurodegeneration of dopaminergic neurons.[101-103]

F-box-containing proteins, sterol regulatory element binding transcription factor 1 (SREBF1), and WD40 domain protein 7 (FBXW7) have been identified as the critical regulators in the PINK/Parkin pathway of mitophagy.[104] Moreover, SREBF1 is also one of the risk factors for sporadic PD,[105] and mitophagy thus may represent a common mechanism linking sporadic and familial PD.[106-108] Mutation in of the F-box domain is reported to be associated with the early-onset autosomal recessive PD. FBXW7

TABLE 1 Major neurodegenerative disease-associated proteins which play roles in mitophagy and their mechanisms

Diseases	Proteins that regulate mitophagy	Mechanisms
PD	PINK1/Parkin	1. Ubiquitinate the OMM proteins, which can thereby be recognized by the autophagy receptor proteins, such as OPTN, p62, and NDP52, and induce mitophagy; 2. Regulate mitochondrial fusion and fission to maintain the homeostasis of mitochondria; 3. Induce Mfn ubiquitination to maintain the MMP
	α-Syn	1. A constituent of the Lewy bodies; 2. Upregulate oxidative stress and impair the mitochondrial function; 3. Activate mPTP through interacting with the VDAC or ANT, disrupt the mitochondrial membrane potential, and initiate mitophagy
	DJ-1	1. Remove the PD-associated aggregated p62; 2. Modulate the elimination of endogenous ROS; 3. Function in parallel with the PINK1/Parkin pathway; 4. Regulate the dimerization of α-Syn
	LRRK2	1. Interact with the regulators of mitochondrial fission and fusion (such as Drp1, OPA1, and Mfn); 2. Regulate the translocation of the CMA complex; 3. Induce the fission of mitochondria through DLP1, then trigger the ULK1-dependent mitophagy; 4. Initiate the JNK signal by interacting with the JIP3 and MKK4/7; 5. Phosphorylate Bcl-2, regulate the mitochondrial depolarization and autophagy; 6. Modulate the mitochondrial calcium uptake
AD	PINK1/Parkin	1. Interact in a process to achieve dynamic equilibrium and regulate mitophagy; 2. Modulate the expression PSEN1, which is the major component of the catalytic center in the APP key enzyme γ-secretase
	Tau	1. Affect the intracytoplasmic translocation of Parkin to the mitochondrial membrane; 2. Regulate Parkin and UCHL1 through the ANT-1-dependent ADP/ATP exchange
	Sirtuins	1. SIRT1 can protect neurons from the Aβ aggregation-induced toxicity; 2. Excessive activation of SIRT2 impairs mitochondrial and microtubule function, leading to mitophagy and Aβ accumulation
HD	PINK1/Parkin	Maintain mitochondrial morphology, ATP levels, and neuronal health, together with the Mfn/MARF, and VDAC/Porin
	GAPDH	Induce the amplified polyglutamine's action, causing mitochondrial dysfunction
	TG2	Cross-link with Htt, resulting in mitochondrial membrane potential loss, and accumulation of abnormal proteins in the brain
	VCP	Combine with LC3 to cause mitophagy, which seems not to be dependent on the PINK1/Parkin pathway
	HAP1	Regulate the neuronal autophagosome transport
ALS	OPTN/TBK1	1. Affect NF-κB activity and intracellular transport; 2. Maintain Parkin-dependent mitophagy homeostasis
	SOD1	Modulate reverse transport of autophagosomes in axons in a PINK1/Parkin pathway-dependent manner
	VCP	Disrupt the mitophagy balance through the PINK1/Parkin pathway

Note. The dysregulation of mitophagy can lead to damaged mitochondria and accumulation of abnormal protein, which are frequently observed in neurodegenerative diseases. This table lists the major mitophagy-linked factors associated with neurodegenerative diseases and the potential pathogenic mechanisms.

Abbreviations: α-Syn, α-synuclein; Aβ, β amyloid protein; AD, Alzheimer's disease; ADP, adenosine diphosphate; ALS, amyotrophic lateral sclerosis; ANT, adenine nucleotide translocator; ANT-1, adenine nucleotide transporter 1; APP, amyloid precursor protein; ATP, adenosine triphosphate; CMA, chaperone-mediated autophagy; DJ-1 (PARK7), Parkinson disease protein 7; DLP1, dynamin-like protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAP1, Htt-associated protein-1; HD, Huntington's disease; JIP3, JNK interacting protein-1; JNK, c-Jun N-terminal kinase; LRRK2, leucine-rich repeat kinase 2; MARF, mitochondrial assembly regulate factors; Mfn, mitofusin; MKK4/7, mitogen-activated protein kinase kinase 4/7; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1; PD, Parkinson's disease; PSEN1, presenilin 1; SIRT1, Sirtuin 1; SIRT2, Sirtuin 2; SOD1, superoxide dismutase 1; TG2, transglutaminase type 2; Trx/Ask1, thioredoxin/apoptosis signal-regulating kinase 1; UCHL1, ubiquitin carboxyl-terminal esterase L1; VCP, valosin-containing protein; VDAC, voltage-dependent anion channel.

is involved in maintaining mitochondria and inducing mitophagy through direct interaction with Parkin, further confirming the significance of mitophagy in the pathogenesis of PD.[109]

Several other genetic mutations, including PINK1, Parkin, DJ-1, LRRK2, and α-Syn, have been linked to familial PD. Mutations in LRRK2 and a-Syn can cause autosomal dominant PD by the gain-of-function and possibly proteotoxic mechanisms, while mutations in PINK1 and Parkin can also induce PD through loss-of-function mechanism. These genes are associated with mitochondrial function, and the corresponding gene products are also involved in mitophagy. Therefore, these proteins may provide mechanistic links between PD with mitophagy.

5.1.1 | PINK1 and Parkin

PINK1 and Parkin are the most extensively studied PD-associated proteins that are involved in mitochondrial function. PINK1 is encoded by the *PARK6* gene and acts as a serine/threonine kinase targeted to mitochondria,[110,111] whereas Parkin is encoded by the *PARK2* gene and is an E3 ubiquitin ligase.[112] Loss of Parkin or PINK1 function results in autosomal recessive PD,[113] and the PINK1-dependent initiation of Parkin recruitment to defective mitochondria is regarded as an important regulatory mechanism in mitophagy. Parkin is normally a cytosolic protein, which is recruited to dysfunctional mitochondria in a PINK1-dependent manner.[114] Parkin will broadly ubiquitinate and degrade the OMM proteins through the ubiquitin-proteasome system.[115]

On dysfunctional mitochondria, ubiquitinated OMM proteins produced by Parkin can be recognized by the autophagy receptors, such as OPTN, p62, and NDP52, which can initiate the autophagy process. PINK1 appears to play an incontrovertible role in mitophagy, but Parkin seems to amplify and promote PINK1 effect but itself is not obligatory, since the recruitment of autophagy receptor would take place and mitophagy would be initiated in the absence of Parkin activation.[85,116] The Parkin-independent events downstream of PINK1 during the mitophagy process remain largely unknown. For instance, the critical substrates and the E3 ligases promoting the mitophagy-inducing ubiquitination signals remain to be fully elucidated.

PINK1 and Parkin are essential for maintaining the homeostasis of mitochondria, which is achieved by regulating mitochondrial fusion and fission[117]; at least in part through Mfn ubiquitination. Loss of PINK1 or Parkin would result in the accumulation of Mfn on OMM and impair the mitophagy process. In contrast, loss of Mfn would result in decreased mitochondrial membrane potential, and Parkin recruitment would be blocked by inhibiting the translocase of the OMM import system (TOM complex).[118] In addition, upregulation of the TOM complex can rescue the mitophagy defect caused by Parkin mutations, suggesting that TOM functions as a significant mediator in PINK1/Parkin-directed mitophagy.

Our recent studies revealed that on damaged mitochondria, the recruitment of cotranslational quality control factors Pelo, ABCE1, and NOT4 to stalled ribosomes results in NOT4-mediated polyubiquitination of ABCE1 and that polyubiquitinated ABCE1 (poly-Ub-ABCE1) provides a molecular signal for recruiting autophagy receptors to initiate mitophagy.[119] We provided evidence supporting that the PINK1 pathway is deployed to stimulate translation of nuclear-encoded respiratory chain (nRCC) mRNAs on mildly damaged mitochondria to promote RC biogenesis and thus mitochondrial repair, revealing a new physiological role of PINK1/Parkin in mitochondrial regulation. However, for severely damaged mitochondria that are beyond repair, the PINK1 pathway is used to direct their clearance by mitophagy. Our results also showed that many of the effects of PINK1 in activating mitophagy, from recruiting C-I30 mRNP to OMM, cotranslational quality control factors and autophagy receptors to OMM-associated mRNPs, to the subsequent activation of mitophagy, could occur in the absence of Parkin. This contrasts the prevailing view that the activation of Parkin and subsequent ubiquitination of a battery of Parkin substrates on MOM is essential for PINK1-activated mitophagy.[120] Consistent with previous studies, our results indicated that though not required, the presence of Parkin provides an amplifying mechanism to promote PINK1-directed mitophagy, as both the recruitment of autophagy receptors and the clearance of damaged mitochondria are accelerated in the presence of Parkin. These studies thus identified some of the earliest molecular signals in recruiting the autophagy machinery to damaged mitochondria and revealed a mechanistic connection between ribosome-associated cotranslational quality control on mitochondrial outer membrane and mitophagy. The identification of altered expression of certain cotranslational quality control factors such as ABCE1 in PD patient brain tissues offered further support for the clinical relevance of these findings.

5.1.2 | α-Syn

α-Syn, a natively unfolded protein, is located at the presynaptic terminal of CNS and participates in the vesicular release; however, it can be detected in some abnormally conformational structures, such as amyloid fibril, oligomer, and protofibril. α-Syn is identified as a key constituent of the Lewy bodies, the cytoplasmic inclusions that signify the pathological features of PD.[121,122] Two autosomal dominant α-Syn gene mutations (namely A53T and A30P) have been associated with PD,[123] In addition to point mutations, some post-translational modifications can also induce the toxic phenotype of protein, including ubiquitination, phosphorylation, oxidation, nitration, and the dopamine-dependent adduct formation.[124]

Studies on the role of α-Syn in PD have revealed a correlation between aberrant α-Syn expression and mitochondrial dysfunction. α-Syn accumulation can elevate oxidative stress and impair the mitochondrial function.[125] Furthermore, overexpressing α-Syn in neurons can cause mitochondrial breakup both in vitro and in vivo, which will eventually lead to cell death.[126] α-Syn accumulation can open the mitochondrial permeability transition pore (mPTP), disrupt the mitochondrial membrane potential, and subsequently initiate mitophagy. In addition, α-Syn can activate mPTP through interacting with the voltage-dependent anion channel

(VDAC) or adenine nucleotide translocator (ANT).[127] A53T mutant of α-Syn can reside in the mitochondrial membrane and impair mitochondrial function as the monomers and oligomers.[128] Importantly, α-Syn can be removed through mitochondrial fission and the activity of Parkin,[129,130] and its role in activating the autophagy of polarized mitochondria demonstrates that there exists certain link between Parkin and α-Syn during mitophagy in PD pathogenesis.

5.1.3 | DJ-1

DJ-1, encoded by the *PARK7* gene, is associated with autosomal recessive PD.[131,132] DJ-1 has been regarded as a redox sensor, with potential roles in mitochondrial homeostasis.[133] The upregulation of DJ-1 can affect macroautophagy in a cell type-dependent manner. For instance, the neuroprotection of DJ-1 in dopaminergic neurons can be suppressed by inhibitors of autophagy and inhibitors of the ERK pathway. DJ-1 can remove PD-associated aggregated p62, while DJ-1 deletion will impede basal autophagy and obstruct the mitochondrial dynamics in mouse embryonic fibroblasts.[134] DJ-1 is localized to mitochondria as a constituent of the thioredoxin/apoptosis signal-regulating kinase 1 (Trx/Ask1) complex, which can modulate the elimination of endogenous ROS.[135,136] DJ-1 deficiency will result in H_2O_2 accumulation in brain mitochondria, elevated oxidative stress level, and death of DA neurons.[137]

In both fibroblasts and neurons, mitochondrial DJ-1 level is elevated following oxidative damage in a Parkin-dependent manner. DJ-1 overexpression can rescue the phenotype of PINK1-knockout but not Parkin-knockout *Drosophila* models, indicating that DJ-1 may function in between PINK1 and Parkin in the same pathway. To further understand the common features of PINK1, Parkin, and DJ-1, bioinformatics examinations have been carried out, which indicate that Miro interacts with PINK1 and Parkin, whereas HSPA4 interacts with PINK1, Parkin, and DJ-1. In addition, there are dozens of common interacting factors among PINK1, Parkin, and DJ-1, most of which are associated with transcriptional regulation. Noteworthily, compared with healthy controls, the expression of these proteins and their associated factors are downregulated in PD patients.[138] DJ-1 can also directly interact with α-Syn, and DJ-1 mutation associated with PD can induce the accumulation of misfolded α-Syn in dopaminergic neurons, whereas the upregulation of DJ-1 can decrease the dimerization of α-Syn.[139]

5.1.4 | LRRK2

Mutations of the leucine-rich repeat kinase 2 (*LRRK2*) gene are associated with familial and sporadic PD and represent the most significant genetic risk factors for PD. LRRK2-dependent neurodegeneration processes may involve vesicular trafficking, cytoskeletal dynamics, autophagy, mitochondria dynamics, and calcium homeostasis. Numerous LRRK2 mutations have been investigated in PD, among them, the G2019S mutation marks the most frequent cause of autosomal dominant familial PD,[140,141] which can also be found in approximately 2% sporadic PD cases. Therefore, elucidating the pathogenicity of LRRK2 may shed light on the molecular mechanisms of PD in general.

Expression of mutant LRRK2 negatively affects mitochondrial health. Endogenous LRRK2 can interact with the regulators of mitochondrial fission and fusion (such as Drp1, OPA1, and Mfn).[142] In PD patient fibroblasts with LRRK2 G2019S mutation, there is increased susceptibility to MPP^+-induced cellular death.[143] The loss of LRRK2 will disrupt the autophagy pathway and promote the production of autophagosomes.[144,145] Although CMA may mediate the degradation of LRRK2, but the high levels of wild-type LRRK2 will obstruct the translocation of the CMA complex, leading to the deficiency of CMA.[146,147]

The expression of LRRK2 G2019S can stimulate mitophagy, and the interaction of LRRK2 with ULK1 may play a vital role in this process. Expression of LRRK2 G2019S can also lead to fission of mitochondria through dynamin-like protein 1 (DLP1), followed by mitochondrial autophagy by the ULK1-dependent process. Apart from ULK1, LRRK2 can also interact with the endogenous JIP3 and MKK4/7, working together to initiate JNK signaling. The JNK signaling pathway is involved in the pathogenic mechanism of mutated LRRK2, as demonstrated by the suppression of LRRK2 G2019S-mediated mitochondrial deficiency by JNK inhibitors.[148] Furthermore, LRRK2 G2019S can also phosphorylate Bcl-2 at Thr 56, and the Bcl-2 mutant will eliminate the mitochondrial depolarization and autophagy induced by LRRK2 G2019s. Therefore, Bcl-2 may function as a bridge linking the LRRK2 G2019S-mediated dysregulation of autophagy with mitochondrial disorders.[149]

Parkinson's disease-related LRRK2 mutations can also activate mitochondrial calcium uptake in cortical neurons and fibroblasts of familial PD patients, presumably through upregulating the expression of mitochondrial calcium uniporter (MCU). Thus, suppressing the ERK1/2-dependent upregulation of MCU can protect from mutant LRRK2-induced dendrite shortening and inhibit MCU-mediated calcium import; consistently, enhancing calcium export from mitochondria is also protective. Therefore, LRRK2-mediated neurodegeneration includes enhanced susceptibility to mitochondrial calcium overload.[150] The toxicity of α-Syn may also be mediated through its interaction with LRRK2 kinase. Common protein interactors may regulate or mediate α-Syn and LRRK2 interaction in PD.[151]

5.2 | Mitophagy and AD

Alzheimer's disease is a progressively developing neurodegenerative disease, which is associated with the main clinical symptoms of memory impairment, aphasia, indiscriminateness, cognitive impairment, visual-spatial impairment, and changes in behavior and personality. Its pathogenic mechanism remains unclear. Extracellular amyloid accumulation will form the senile plaques, and such cellular changes, together with the intracellular hyperphosphorylation of microtubule-associated tau protein, account for the pathological hallmarks of AD.[152]

Recent studies have found that the development of AD is closely correlated with mitochondrial autophagy defects.[153,154] In a transgenic mouse model of AD, the amyloid-β protein is accumulated, accompanied by a cascade of upregulated mRNA levels of mitochondrial autophagy-associated proteins, such as p62, PARK2, DNM1L, BECN1, BNIP3, PINK1, and LC3. In humans, mice, and even nematodes, Aβ can induce UPR^{mt} and mitophagy in a conserved manner, but the specific mechanism remains to be fully defined. The increases in UPR^{mt} and mitophagy are beneficial, which have been shown to contribute to delaying disease progression and boosting the survival in nematodes.[155] Moreover, Disrupted-in-Schizophrenia-1 (DISC1) has been identified to act as a newly discovered mitophagy receptor. The downregulation of DISC1, as well as Aβ accumulation in AD, will be mutually reinforced, thus creating a vicious circle. Additionally, DISC1 has been shown to protect synapses from Aβ accumulation-associated toxicity through promoting mitophagy.[156]

5.2.1 | PINK1/Parkin

The PINK1/Parkin pathway is a hotspot in research concerning the effect of mitophagy on AD pathology.[157,158] In neurons expressing the human amyloid precursor protein (hAPP), the AD-related mitochondrial stress is found to markedly promote Parkin-dependent mitophagy. Under normal conditions, more Parkin would be recruited to mitochondrial membrane in AD neurons than in healthy neurons, which has been confirmed in AD patient brain samples. This phenotype can be manifested by the enhanced Parkin-mediated mitophagy and decreased intracellular Parkin concentration during disease progression. In patient fibroblast models, upregulating Parkin expression and enhancing autophagy can restore mitochondrial membrane potential, reduce PINK1 expression, and hinder mitochondrial accumulation. It is also found that in AD, Parkin could reversely regulate PINK1 through a special way, and the two proteins could interact to achieve dynamic equilibrium. Specifically, Parkin can upregulate the mRNA and protein expression levels of PSEN1, which is one of the major components of the catalytic center in the APP key enzyme γ-secretase. The APP intracellular domain (AICD) is produced by APP through γ-secretase-mediated cleavage, and it can act on FOXO3, eventually upregulating PINK1 level and downregulating the SQSTM/TIMM/TOMM signaling.[159]

5.2.2 | Tau

In the case of human wild-type full-length Tau (hTau), its intracellular accumulation will affect the mitochondrial membrane potential, thereby inducing mitochondrial maladjustment and dysfunction. Besides, it was recently found that hTau can prevent the intracytoplasmic translocation of Parkin to the mitochondrial membrane and inhibit mitophagy in nematode models, while such effect is independent of the changes in membrane potential. On the other hand, the NH2-terminal fragment of tau can regulate Parkin and UCHL-1 through inhibiting the ANT-1-dependent ADP/ATP exchange, suppress mitochondrial autophagy, and mediate synaptic degeneration in AD.[160]

5.2.3 | Sirtuins

There are also other genes and proteins that can act as a bridge between mitophagy and the progression of AD. The sirtuin family is comprised of seven sirtuins. Three of them have been confirmed to be the mitochondrial sirtuins that are closely related to the mitochondrial performance.[161] Resveratrol has been indicated to upregulate SIRT1 expression, thereby protecting neurons from the Aβ aggregation-induced toxicity. It is shown that the antiinflammatory and antioxidant properties of resveratrol can delay the development of AD, and resveratrol can remarkably enhance mitochondrial autophagy.[162] Resveratrol can also markedly reverse Aβ effects, manifested by elevating the levels of LC3-II/LC3-I, Parkin, and Beclin 1. Additionally, it can reduce the positioning of LC3 and TOMM20, and in the meantime, it modulates BNIP3- and NIX-related processes.[163-165] In AD, excessive activation of the tubulin deacetylase SIRT2 will result in mitochondrial dysfunction and the ensuing mitophagy. On the other hand, pharmacological inhibition or gene knockdown of SIRT2 will improve microtubule function, restore mitochondrial homeostasis, improve autophagy, and reduce Aβ accumulation.

In general, accumulating evidences have verified the presence of mitochondrial dysfunction and abnormal mitochondrial autophagy in the brains of AD patients, which are closely related to disease occurrence and progression. By regulating mitophagy and improving mitochondrial function, it may help delay the pathological process, which is of great significance for the eventual establishment of clinical treatment strategies.

5.3 | Mitophagy and HD

HD is an autosomal dominant neurodegenerative disease, which is frequently seen in middle-aged people. Its clinical symptoms primarily feature dance-like movements, followed by cognitive impairment, and even loss of behavioral and language ability. The progression of HD follows an intractable course, and the survival time for patients is generally 10-20 years. The main cause of HD is the mutation in the huntingtin (Htt) gene located on chromosome 4. Specifically, the CAG repeat in exon 1 of Htt is expanded, resulting in expansion of N-terminal polyglutamine of the mutant Htt protein. Mutant Htt protein gradually accumulates in cells to form the macromolecular aggregation, which will induce cytotoxicity. Mutant Htt can interfere with the normal function of nerve cells through a number of mechanisms, including mitochondrial dysfunction, oxidative stress, amino acid metabolism, protein transport, and the apoptosis signaling. The presence of mHtt can result in neurological abnormalities or regression.[166-168]

Increasing evidence suggests that mutant Htt can damage mitochondria, leading to energy metabolism disorders, oxidative stress, and release of proapoptotic factors.[18,169] The aberrant morphology of mitochondria can be observed in the *Drosophila* model of HD. In

addition, mutant Htt is found to cause formation of spherical mitochondria in a nonapoptotic state, and it can also repress mitophagy, resulting in the impaired mitochondrial clearance.[170]

5.3.1 | PINK1/Parkin

In the *Drosophila* model of HD, increased PINK1 expression can suppress the mitochondrial morphological abnormalities and accumulation, while overexpression of PINK1 reduces neuronal vacuolization, restores ATP levels, and reduces mortality in adult *Drosophila*. These results suggest that PINK1 can protect neurons against mutant Htt toxicity. However, the effect of PINK1 is dependent on Parkin activity, which requires the involvement of the Mfn/mitochondrial assembly regulate factors (MARF) and VDAC/Porin. Similar results can be obtained in the mouse model of HD, in which mutant Htt can reduce the targeting of mitochondria to autophagosomes, which can be reversed by the overexpression of PINK1.[18]

5.3.2 | GAPDH

Polyglutamine expansion is an important pathogenic mechanism of HD. Recent studies revealed that the amplified polyglutamine can cause mitochondrial dysfunction, which is mainly characterized by morphological abnormalities, blockade of respiratory function, reduced ATP production, and the release of proapoptotic factors. Such mitochondrial damage is at least partly ascribed to the inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on mitochondria. This is followed by the inhibition of GAPDH-mediated mitophagy and the accumulation of impaired mitochondria in cells. GAPDH selectively binds to mitochondria, and overexpression of GAPDH can restore mitochondrial function.[171]

5.3.3 | Transglutaminase type 2

Transglutaminase type 2 (TG2) is closely related to mitochondrial clearance and homeostasis. Its expression in the brain of HD patients is elevated, and it cross-links with Htt, resulting in mitochondrial membrane potential loss, accumulation of abnormal proteins, and neuronal apoptosis in the brain. On the other hand, pharmacological inhibition or gene knockdown of TG2 can protect against neurodegeneration in HD, and TG2 may be one of the causes of progressive death of HD neurons.[172]

5.3.4 | Valosin-containing protein

Recent studies have shown that in the HD model, valosin-containing protein (VCP) is localized on mitochondria where it specifically binds mutant Htt,[173] while VCP accumulation can lead to excessive activation of mitophagy, thus inducing neuronal damage. This effect is caused by the interaction of its LIR with LC3, and it seems not to be dependent on the PINK1/Parkin pathway, but the detailed mechanism remains unclear. A peptide HV-3 can block VCP translocation to mitochondria and inhibit excessive mitophagy damage. It has been shown to exhibit the neuron protective effect in the HD model. Therefore, inhibiting the interaction of VCP with mutant Htt may be a new strategy for treating HD.[173]

Impaired axonal transport of autophagosomes has also been suggested to be responsible for the inability of abnormal mitochondria to be cleared in HD. The Htt-associated protein-1 (HAP1) and Htt are shown to regulate neuronal autophagosome transport.[170] Depletion of Htt increases the autophagosomes containing mitochondrial fragments, indicating that mitochondrial clearance is reduced. However, the authors found that Htt would not alter the flux of mitophagy. Inhibiting the α-tubulin deacetylase HDAC6 is also considered a potential avenue for treating HD, but the mechanism remains unclear, and studies suggest that HDAC6 may block mitophagy.[174] Various studies have revealed reduced mitophagy in the brain of HD patients, which lead to subsequent dysfunction.[171] Currently, developing treatment strategies for HD by rebuilding stable mitophagy balance has attracted great interests.

5.4 | Mitophagy and ALS

ALS is a neurodegenerative disease that develops in adulthood, accompanying the loss of motor neuron disease (MND) and upper motor neuron (UMN). The pathological hallmark of ALS is progressive motor dysfunction, but the sensory function is found to be unaffected. Nonetheless, it has been found in recent years that the neurodegeneration in ALS is not limited to the motor system; instead, it has involved the sensor, linguistic, behavioral, and other cognitive domains. Some patients may have mild cognitive impairment and even significant frontotemporal dementia.

The pathogenesis of ALS has been extensively explored. More than 30 gene mutations have been reported to be associated with ALS so far, and SOD1, OPTN, TBK1, VCP, and C9ORF72 have become the research hotspots. In ALS models, the accumulation of damaged mitochondria can be detected by live cell imaging. Mitochondrial homeostasis can be reconstituted by inhibiting OPTN or TBK1 mutations, as well as pharmacological inhibition or genetic knockdown of PINK1 or Parkin. Altering TBK1/OPTN can significantly improve neuronal function and block disease progression. These data support the potential role of mitochondrial autophagy in ALS.[89,91,175]

5.4.1 | OPTN/TBK1

Three ubiquitin-LC3-binding autophagy receptors involved in mitophagy have been studied, including OPTN, CALCOCO2, and TAX1BP1. OPTN mutations are well known to underlie the pathogenesis of glaucoma and ALS.[176] It was found that the time course of recruitment of these receptors to the mitochondria is similar, but only OPTN can trigger the subsequent formation of autophagosomes around damaged mitochondria. Moreover, OPTN mutations have been shown in previous studies to enhance NF-κB activity and impede intracellular transport. Besides, mutations are suggested to activate the inflammatory factors, which also contribute to disease progression.[177]

As found in recent studies, OPTN will translocate to damaged mitochondria, which is dependent on Parkin ubiquitination of mitochondria.[175] Time course studies during mitophagy reveal that the recruitment of Parkin, that of OPTN, as well as the formation of autophagosomes occur at 30, 45, and 60 min after injury, respectively. Besides, OPTN can bind with mitochondria transiently in the absence of Parkin. But such binding would be more stable in the presence of Parkin. Subsequently, DFCP1 can translocate to mitochondria and recruit LC, and the LIR of OPTN will mediate the formation of autophagosomes around the scathing mitochondria, indicating that OPTN is important in the Parkin-mediated mitophagy. However, this process will be hindered, and mitochondrial accumulation will be impaired if the endogenous OPTN is depleted. Notably, the wild-type mice with OPTN siRNA resistance can withstand such damage, but mice with the ALS-related OPTN mutation (E478G) cannot. The ALS-related OPTN UBAN mutant has been utilized to block the translocation of OPTN to the mitochondria, which will lead to abnormal mitophagy. The result thus links OPTN with ALS through the mitochondrial degradation efficiency, demonstrating the significance of mitophagy to ALS.

The effect of OPTN translocation to mitochondria to induce mitophagy is consistent with the recruitment of its upstream kinase TBK1. Specifically, the OPTN-mediated mitophagy depends on the phosphorylation of its serine 177 by TBK1. Similar to OPTN, TBK1 mutations can also cause ALS. Pharmacological inhibition of TBK1 or expression of ALS-associated TBK1 mutants can reduce the mitophagic efficiency of damaged mitochondria. In conclusion, both OPTN and its upstream protein TBK1 are required for normal mitophagy homeostasis.[178] As shown in these studies, TBK1 phosphorylates OPTN, enhancing its ability to bind to ubiquitinated mitochondria, suggesting that TBK1 may also be one of the potential targets for treating ALS.[94]

Furthermore, evidence suggests that the TBK1/OPTN axis is linked to the PINK1/Parkin pathway, which can mediate autophagosome production and the clearance of impaired mitochondria. Both inhibition of OPTN or TBK1 and knockdown of PINK1 or Parkin can ameliorate mitophagy in ALS. However, the knockout of PINK1 or Parkin would exert no significant neuroprotection. Reasons may be that PINK1/Parkin mediates the production of autophagosomes and can be seen anywhere in the neuron, but the engulfment of mitochondria by autophagosomes is dependent on the TBK1/OPTN pathway.[179]

5.4.2 | SOD1

SOD1 is one of the most extensively investigated ALS-related genes. Its mutations cause accumulation of hydroxyl radicals, trigger cytotoxicity, and contribute to ALS. Notably, it has recently been reported that SOD1 may indirectly participate in regulating the mitochondrial homeostasis in ALS.[180,181]

On the other hand, it is found that in the SOD1 model of ALS, the flux of mitophagy is decreased. Mitochondria quality control (MQC) can restore mitophagy and reduce the accumulation of impaired mitochondria, which may be ascribed to the potential effect of SOD1 in blocking the reverse transport of autophagosomes in axons. It is suggested that SOD1 relies on Parkin to reduce the rate-limiting factor mitochondrial Rho-GTPase 1 (Miro1), whereas overexpression of Miro1 and inhibition of PINK1 can reverse the autophagosome trafficking defect resulted from the SOD1 mutations. It is suggested that mutant SOD1 would impair axonal transport in a PINK1/Parkin pathway-dependent manner.[182] In the G93A mutant SOD 1 transgenic (SOD1-G93A) mouse model, mitochondria can recruit P62 and activate mitophagy. In that model, the mitochondrial defects are manifested as a variety of protein changes, such as ubiquitin ligase Parkin, mitochondrial biogenesis protein PGC-1α, and Miro1. Knocking down Parkin can reverse such negative effect and improve the pathological symptoms of ALS. But the effect is not sustained and may be compensated by other ubiquitinating enzymes.[169,183,184]

In addition, VCP also exhibits some properties similar to OPTN; for instance, it relies on the Parkin-mediated ubiquitination to be recruited to the mitochondria and is involved in mitophagy. The ALS-related VCP mutations will disrupt the mitophagy balance through the PINK1/Parkin pathway, thereby affecting the clearance of abnormal mitochondria.[185]

At present, the pathogenesis of ALS remains to be fully understood, and the existing hypotheses include oxidative stress, mitochondrial dysfunction, excitotoxicity, and neuroinflammation.[186-188] Notably, research on mitophagy, inflammation, and their interaction has become a hotspot. Improving mitochondrial autophagy and restoring mitochondrial homeostasis may offer potential treatment for ALS.

6 | CONCLUSIONS

Neurodegenerative diseases are associated with protein turnover, and protein aggregation is involved in the cellular pathology of many neurodegenerative diseases. Mitophagy can partially account for the mechanisms of cellular homeostasis; therefore, appropriate mitophagy level is of great significance to reduce the aggregation of abnormal proteins and to stimulate organelle removal. For the sake of protecting neurons, it is necessary to maintain the mitochondrial function and promote the degradation of damaged mitochondria. The regulation of mitophagy is suggested to be one of the therapeutic strategies for some neurodegenerative diseases. The activation of mitophagy has been shown to improve neurodegenerative diseases phenotypes and offer neuroprotection. Several therapeutic tools have been confirmed to increase mitophagy, such as the regulators of PINK1/parkin, metformin, and resveratrol.[189] In recent experiments, some drugs are also thought to delay the disease progression, which target the sirtuins family. Sirtuin activating compounds (STACS) or NAD precursors such as NR/NMN can increase mitophagy through regulating sirtuins.[189,190] Many natural compounds with bioactivation are gradually being discovered. Some antibiotics or plant ingredients also induce mitophagy, such as

actinomycetes.[191] It is associated with mitochondrial autophagy in neural stem cells, which may be mediated by ribosome depletion.[192]

Nevertheless, both excessive and reduced mitophagy may be harmful, making mitophagy a double-edged sword. Mitochondrial autophagy plays an important role in the self-maintenance of the nervous system, but only the role of PINK1/Parkin pathway in the regulation of neurodegenerative diseases has been well specified so far, and some results are still controversial. The current results only partially reveal the role of mitophagy mediated by the PINK1/Parkin pathway in PD, while there are few studies on AD and HD, and many questions have not been clarified.[193,194] NIX/BNIP3L and FUNDC1 mainly regulate mitochondrial autophagy under hypoxic conditions, but the regulation of mitochondrial autophagy under hypoxic and ischemic conditions needs to be further explored.[195]

Consequently, simply elevating the mitophagy level is not a feasible approach, and there are still some future challenges; for instance, how to optimize the mitophagy activity for neuron protection to treat neurodegenerative diseases, how to elucidate the common molecular mechanisms regarding the pathogenesis of neurodegenerative diseases, and how to clarify the interactions among mitophagy, mitochondrial metabolism, and mitochondrial dynamics. Researches on the molecular mechanisms related to mitophagy should be strengthened, and technologies that can visually and real-time monitor mitochondrial morphological changes should be developed. In addition, more specific agents that target mitophagy can be applied to cultured neurons or animal models of neurodegenerative diseases to observe the relationship between mitophagy and diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CELL DEATH & AUTOPHAGY

Mechanism and medical implications of mammalian autophagy

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Abstract | Autophagy is a highly conserved catabolic process induced under various conditions of cellular stress, which prevents cell damage and promotes survival in the event of energy or nutrient shortage and responds to various cytotoxic insults. Thus, autophagy has primarily cytoprotective functions and needs to be tightly regulated to respond correctly to the different stimuli that cells experience, thereby conferring adaptation to the ever-changing environment. It is now apparent that autophagy is deregulated in the context of various human pathologies, including cancer and neurodegeneration, and its modulation has considerable potential as a therapeutic approach.

The discovery of lysosomes by Christian de Duve[1] more than 60 years ago marked the birth of a new research field and earned its trailblazer a Nobel Prize in Physiology or Medicine in 1974. The delivery of heterogenic intracellular material to lysosomal digestion was termed 'autophagy' (Greek for 'self-eating') by de Duve as early as 1963, but consequent research on autophagy did not receive much attention for more than 30 years. Major achievements at that period focused on the tight regulation of autophagy by nutrient availability[2], while the physiological relevance and manner of lysosomal delivery remained unknown. Then, Yoshinori Ohsumi's laboratory conducted a genetic screen to dissect the process in yeast[3], identifying 15 autophagy-related proteins (ATGs) essential for autophagic delivery of cargo to the vacuole (the counterpart of the lysosome in yeast) (FIG. 1; TABLE 1). From that point onwards, the field explosively increased in knowledge, and the fundamental physiological importance of autophagy for human health and disease was uncovered. In 2016, Ohsumi was awarded a Nobel Prize in Physiology or Medicine for his discovery of mechanisms of autophagy.

We now know that autophagy is an adaptive process that occurs in response to different forms of stress, including nutrient deprivation, growth factor depletion, infection and hypoxia. We also understand much better how the autophagic machinery is regulated and selects cargo and how its perturbation affects cellular and organismal function. The main function of autophagy is to provide nutrients for vital cellular functions during fasting and other forms of stress; thus, autophagy has long been considered a nonselective process. However, autophagy was more recently shown to selectively eliminate unwanted, potentially harmful cytosolic material, such as damaged mitochondria or protein aggregates (a process known as selective autophagy; see BOX 1), thereby acting as a major cytoprotective system. Intriguingly, autophagy is also used by cells to secrete cytoplasmic constituents. Accordingly, autophagic activity modulates many pathologies, including neurodegeneration, cancer and infectious diseases, thus also placing autophagy under the spotlight of pharmacologists and clinicians.

In this Review, we summarize the molecular mechanisms and regulation of mammalian autophagy and describe their involvement in several pathological conditions. We also discuss current strategies, limitations and challenges involved in targeting the pathway in cancer and neurodegenerative diseases where most knowledge has accumulated over the past years.

Mechanism of autophagy

Induction of autophagy results in recruitment of ATGs to a specific subcellular location termed the phagophore assembly site (PAS) and nucleation of an isolation membrane that forms a cup-shaped structure termed the phagophore (FIG. 1). Gradual elongation of the curved isolation membrane results in expansion of the phagophore into a sphere around a portion of the cytosol. The isolation membrane eventually seals into a double-membraned vesicle, termed the autophagosome, thereby trapping the engulfed cytosolic material as autophagic cargo. After clearance of most ATGs and delivery along microtubules to the lysosome, the outer membrane of the autophagosome fuses with the lysosomal membrane to form an autolysosome. This fusion results in the release of a single-membrane autophagic body into the lysosomal lumen, which is followed by the degradation of the autophagic body together with its cargo by the autolysosomal hydrolytic milieu[4–6].

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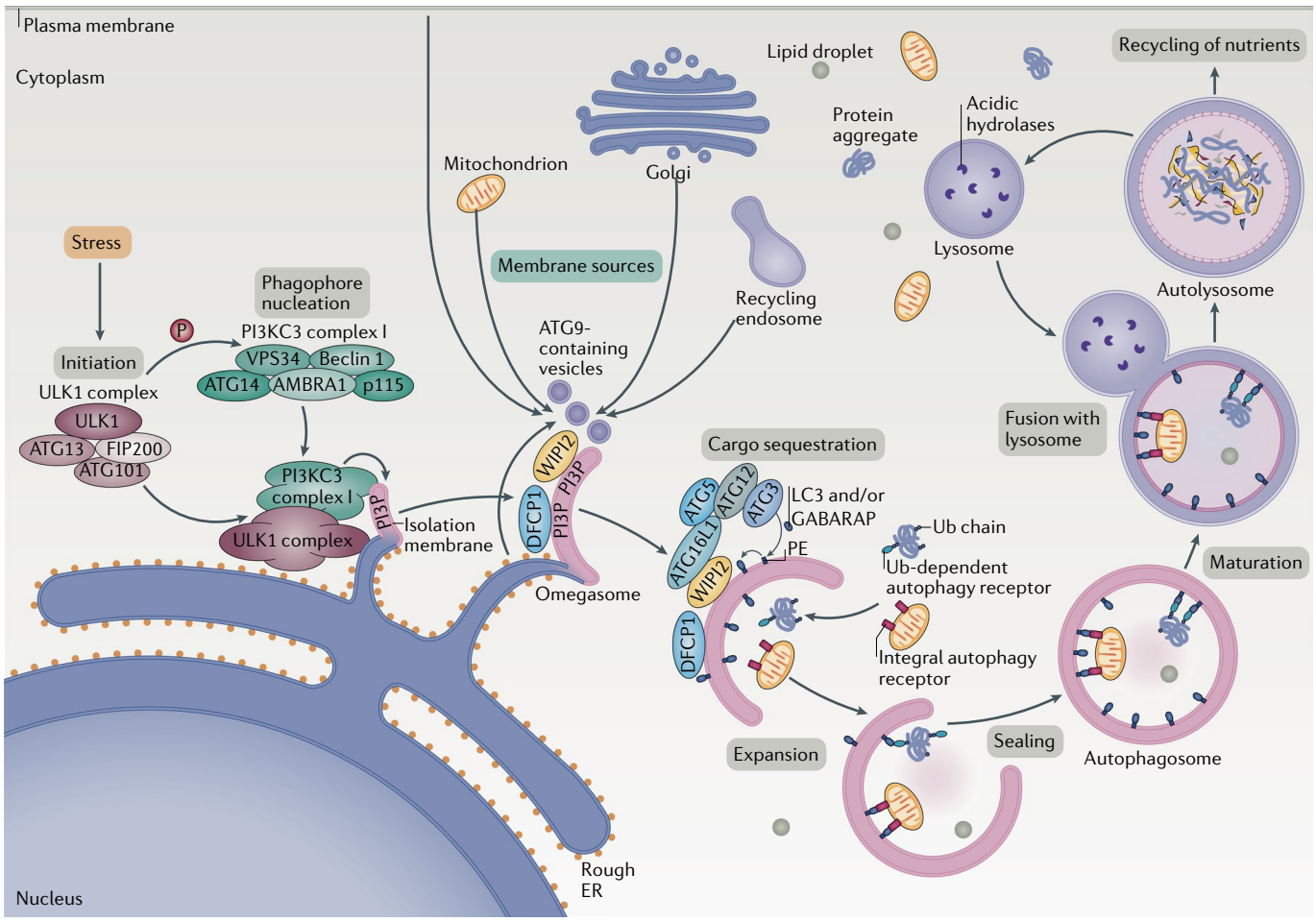


Fig. 1 | **Overview of the autophagy process.** Signals that activate the autophagic process (initiation) typically originate from various conditions of stress, such as starvation, hypoxia, oxidative stress, protein aggregation, endoplasmic reticulum (ER) stress and others. The common target of these signalling pathways is the Unc-51-like kinase 1 (ULK1) complex (consisting of ULK1, autophagy-related protein 13 (ATG13), RB1-inducible coiled-coil protein 1 (FIP200) and ATG101), which then triggers nucleation of the phagophore by phosphorylating components of the class III PI3K (PI3KC3) complex I (consisting of class III PI3K, vacuolar protein sorting 34 (VPS34), Beclin 1, ATG14, activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1) and general vesicular transport factor (p115)), which in turn activates local phosphatidylinositol-3-phosphate (PI3P) production at a characteristic ER structure called the omegasome. PI3P then recruits the PI3P effector proteins WD repeat domain phosphoinositide-interacting proteins (WIPIs; here WIPI2) and zinc-finger FYVE domain-containing protein 1 (DFCP1) to the omegasome via interaction with their PI3P-binding domains. WIPI2 was recently shown to bind ATG16L1 directly, thus recruiting the ATG12~ATG5–ATG16L1 complex that enhances the ATG3-mediated conjugation of ATG8 family proteins (ATG8s), including microtubule-associated protein light chain 3 (LC3) proteins and γ-aminobutyric acid receptor-associated proteins (GABARAPs) to membrane-resident phosphatidylethanolamine (PE), thus forming the membrane-bound, lipidated forms; for example, in this conjugation reaction, LC3-I is converted into LC3-II — the characteristic signature of autophagic membranes. ATG8s not only further attract components of the autophagic machinery that contain an LC3-interacting region (LIR) but also are required for elongation and closure of the phagophore membrane. Moreover, in selective autophagy, LC3 is critically involved in the sequestration of specifically labelled cargo into autophagosomes via LIR-containing cargo receptors. Several cellular membranes, including the plasma membrane, mitochondria, recycling endosomes and Golgi complex, contribute to the elongation of the autophagosomal membrane by donating membrane material (part of these lipid bilayers is delivered by ATG9-containing vesicles, but the origin of the rest of the lipid bilayer is currently unknown). Sealing of the autophagosomal membrane gives rise to a double-layered vesicle called the autophagosome, which matures (including stripping of the ATG proteins) and finally fuses with the lysosome. Acidic hydrolases in the lysosome degrade the autophagic cargo, and salvaged nutrients are released back to the cytoplasm to be used again by the cell. Ub, ubiquitin.

The autophagic pathway and core autophagy proteins. The so-called core ATG proteins essential for autophagosome formation and lysosomal delivery of autophagic cargo are grouped by their functional and physical interactions into five complexes[7] (see also TABLE 1): (i) the ULK1 (Unc-51-like kinase 1) complex — the serine/threonine protein kinase ULK1, RB1-inducible coiled-coil protein 1 (FIP200; also known as RB1CC1), ATG13 and ATG101; (ii) ATG9 — the sole integral, transmembrane core ATG; (iii) the class III PI3K (PI3KC3) complex — the catalytic subunit vacuolar protein sorting 34 (VPS34) that converts PI into PI-3-phosphate (PI3P), Beclin 1 and general vesicular transport factor p115, joined by ATG14 in PI3KC3 complex I (PI3KC3–C1) or UV radiation resistance-associated gene protein (UVRAG) in complex II (PI3KC3–C2); (iv) WIPI (WD repeat domain phosphoinositide-interacting) proteins and their functional, optionally physical interaction partner ATG2;

Table 1 | **Key autophagic factors and their regulation**

Protein	Function	Mechanisms of regulation
Initiation and phagophore nucleation		
ULK1 and ATG1	Serine/threonine kinase; initiates autophagy by phosphorylating components of the autophagy machinery	Stress and nutrients (via mTORC1, AMPK and LKB1); TFEB and several miRNAs
FIP200	Component of ULK complex (possibly scaffolding function)	ULK1 and miRNAs
ATG13	Adaptor mediating the interaction between ULK1 and FIP200; enhances ULK1 kinase activity	ULK1, mTORC1 and AMPK
ATG101	Component of ULK complex; recruitment of downstream ATG proteins	ULK1
VPS34	Catalytic component of PI3KC3–C1; generates PI3P in the phagophore and stabilizes the ULK complex	AMPK, ULK1 and p300 (acetylation)
Beclin 1	Promotes formation of PI3KC3–C1 and regulates the lipid kinase VPS34	Activation: AMPK, ULK1, MAPKAPK2, MAPKAPK3, DAPK and UVRAG; inhibition: BCL-2, AKT and EGFR
ATG14	PI3KC3–C1 targeting to the PAS and expanding phagophore	PIPKIγI5 and mTORC1
ATG9	Delivery of membrane material to the phagophore	ULK1 complex
WIPI2	PI3P-binding protein that recruits ATG12~ATG5–ATG16L to the phagophore; retrieval of ATG9 from early autophagosomal membranes	TFEB (positive transcription regulator) and ZKSCAN3 (negative transcription regulator)
Phagophore expansion		
ATG4	Cysteine protease that processes pro-ATG8s; also, deconjugation of lipidated LC3 and ATG8s	ULK1 and ROS
ATG7	E1-like enzyme; activation of ATG8; conjugation of ATG12 to ATG5	miRNAs
ATG3	E2-like enzyme; conjugation of activated ATG8s to membranal PE	miRNAs
ATG10	E2-like enzyme that conjugates ATG12 to ATG5	miRNAs
ATG12~ATG5–ATG16L	E3-like complex that couples ATG8s to PE	CSNK2
PE-conjugated ATG8s	Scaffold for assembly of the ULK1 complex; supports membrane tethering and hemifusion events for phagophore expansion	ULK1, PKA, ATG4 and mTOR
ATG9	Delivery of membrane material to the phagophore	ULK1
Cargo sequestration		
Ubiquitin	Cargo labelling	PINK (phosphorylation)
Cardiolipin and ceramide	Cargo labelling	Phosphorylation
p62	Autophagy receptor	ULK1 and TBK1
OPTN	Autophagy receptor	TBK1
NBR1	Autophagy receptor	TBK1
NDP52	Autophagy receptor	TBK1
PE-conjugated LC3	Interaction with autophagy receptors; also phagophore expansion and sealing	ULK1, PKA, ATG4 and mTOR
Membrane sealing		
LC3s and GABARAPs	Unclear	Unclear; might involve phosphorylation and acetylation events
Autophagosome maturation		
ATG4	Removal of ATG8s from the surface of the autophagosome	Unknown
PE-conjugated LC3s and GABARAPs	Linking the autophagosome to microtubule-based kinesin motor	Unclear; might involve phosphorylation and acetylation events

Table 1 (cont.) | **Key autophagic factors and their regulation**

Protein	Function	Mechanisms of regulation
Fusion with the lysosome		
PE-conjugated LC3s and GABARAPs	Mediates autophagosome–lysosome fusion upon phosphorylation through PLEKHM1 and HOPS	STK3 and STK4
ATG14	Promotes SNARE-driven membrane fusion	Unknown
Rab GTPase RAB7	Unclear	Unknown

ATG, autophagy-related protein; AMPK, 5′ AMP-activated protein kinase; CSNK2, casein kinase 2; DAPK, death-associated protein kinase; EGFR, epidermal growth factor receptor; FIP200, RB1-inducible coiled-coil protein 1; GABARAP, γ-aminobutyric acid receptor-associated protein; HOPS, homotypic fusion and protein sorting; LC3, light chain 3; LKB1, liver kinase B1; MAPKAPK, MAPK-activated protein kinase; miRNA, microRNA; NBR1, neighbour of *BRCA1* gene; NDP52, nuclear dot protein 52; OPTN, optineurin; p62, also known as SQSTM1; p300, histone acetyltransferase 300; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PI3P, phosphatidylinositol-3-phosphate; PINK, PTEN-induced putative kinase 1; PIPKIγi5, type Iγ PIP kinase isoform 5; PI3KC3, class III PI3K; PKA, protein kinase A; PLEKHM1, pleckstrin homology domain-containing protein family member 1; RAB, Ras-related protein; ROS, reactive oxygen species; STK, serine/threonine protein kinase; TBK1, TANK-binding kinase 1; TFEB, transcription factor EB; ULK1, Unc-51-like kinase 1; UVRAG, ultraviolent irradiation resistance-associated gene; VPS34, class III PI3K vacuolar protein sorting 34; WIPI2, WD repeat domain phosphoinositi de-interacting protein 2; ZKSCAN3, zinc-finger protein with KRAB and SCAN domains 3.

and (v) two ubiquitin (Ub)-like proteins and covalent conjugation targets (and their activation and conjugation machinery, see below): the Ub-like ATG12 conjugates with ATG5 (ATG12~ATG5), where ~ denotes conjugation, which further establishes a complex with ATG16L (ATG12~ATG5–ATG16L), and Ub-like ATG8 family proteins (ATG8s), which include the light chain 3 (LC3) subfamily (also known as microtubule-associated proteins 1 A/1B LC3, MAP1LC3): LC3A, LC3B, LC3C and the γ-aminobutyric acid receptor-associated protein (GABARAP) subfamily (GABARAP, GABARAPL1, GATE-16/GABARAPL2), which form conjugates with membrane-resident phosphatidylethanolamine (PE).

Induction and phagophore nucleation. Phagophores are nucleated at the PAS on endoplasmic reticulum (ER)-emanating membrane domains termed 'omegasomes' that are PI3P-rich and marked by the PI3P-binding protein zinc-finger FYVE domain-containing protein 1 (DFCP1; also known as ZFYVE1). However, ER-mitochondria and ER-plasma membrane contact sites[8,9] as well as other organelles, such as the Golgi complex, plasma membrane and recycling endosomes, were also recently implicated as PASs (see recent reviews[4]), possibly reflecting different experimental tools or the contribution of different intracellular membrane sources to autophagosome formation, which may be cell dependent and/or context dependent. Nucleation of the phagophore membrane is an intricate process, and its molecular details are still not completely understood. According to current understanding, phagophore formation involves the cooperative PAS formation, activation of the ULK1 complex and the PI3KC3–C1, possibly in concert with the activation of localized PI synthase. These events are accompanied by the recruitment of ATG9-containing vesicles generated by the secretory pathway to the PAS, which may deliver additional lipids and proteins contributing to membrane expansion[10–12]. Accordingly, activation of the ULK1 and PI3KC3–C1 are immediate responses to autophagy induction.

The most characterized trigger for induction of autophagy is deprivation of amino acids, which results in inhibition of the master cell growth regulator serine/threonine kinase mTOR[13,14]. mTOR is found in two distinct protein complexes, mTORC1 and mTORC2, but only mTORC1 directly regulates autophagy[15]. In high-nutrient conditions, ATG13 and ULK1 are both directly bound and phosphorylated by mTORC1 and remain inactive in this phosphorylated form[16]. Upon starvation, mTORC1 sites on ULK1 are dephosphorylated and ULK1 dissociates from mTORC1. Concomitantly, ULK1 undergoes autophosphorylation, followed by phosphorylation of ATG13 and FIP200. ULK1 is activated upon dissociation from mTOR after autophosphorylation, followed by phosphorylation of ATG13 and FIP200 by ULK1 (REFS[16,17]). Another key regulator of autophagy is TFEB (transcription factor EB), which is a master transcription factor controlling cellular clearance. Like ATG13 and ULK1, TFEB is negatively regulated by mTORC1 and released upon starvation to regulate the expression of genes involved in lysosomal biogenesis and lipid catabolism[18]. TFEB family members control autophagy by mTORC1 lysosomal recruitment and activity by directly regulating expression of the mTOR-activating Rag GTPase complex component Ras-related GTP-binding protein D (RagD)[19], thus providing a feedback circuit to balance the cellular metabolic state.

Autophagy may also be induced upon declining cellular energy levels, as in glucose starvation, sensed through the ATP:AMP ratio by cell homeostasis regulatory kinases 5′ AMP-activated protein kinase (AMPK) and serine/threonine-protein kinase STK11 (LKB1)[20]. LKB1 activates autophagy through AMPK by inhibition of mTORC1 indirectly via activation of the TSC2 (tuberous sclerosis 2) complex and possibly directly by phosphorylation of Raptor[21]. As TSC2 is regulated by interaction with WIPI3 and FIP200 (REF.[22]), involvement of the LKB1–AMPK–TSC2 axis in CREB-regulated transcription coactivator 1 (TORC1; also known as CRTC1) regulation provides a feedback control on autophagy induction. Because TSC2 is regulated by WIPI3 and FIP200, involvement of LKB1-AMPK-TSC2 axis in mTORC1 regulation allows coordination between autophagy induction and autophagosome formation. AMPK-mediated induction of autophagy can also bypass mTOR by directly inducing phosphorylation of ULK1, VPS34 and Beclin 1 (REF.[23]).

Contact sites
Interorganellar connections with distinct biochemical properties and a characteristic set of proteins that function as signalling hot spots.

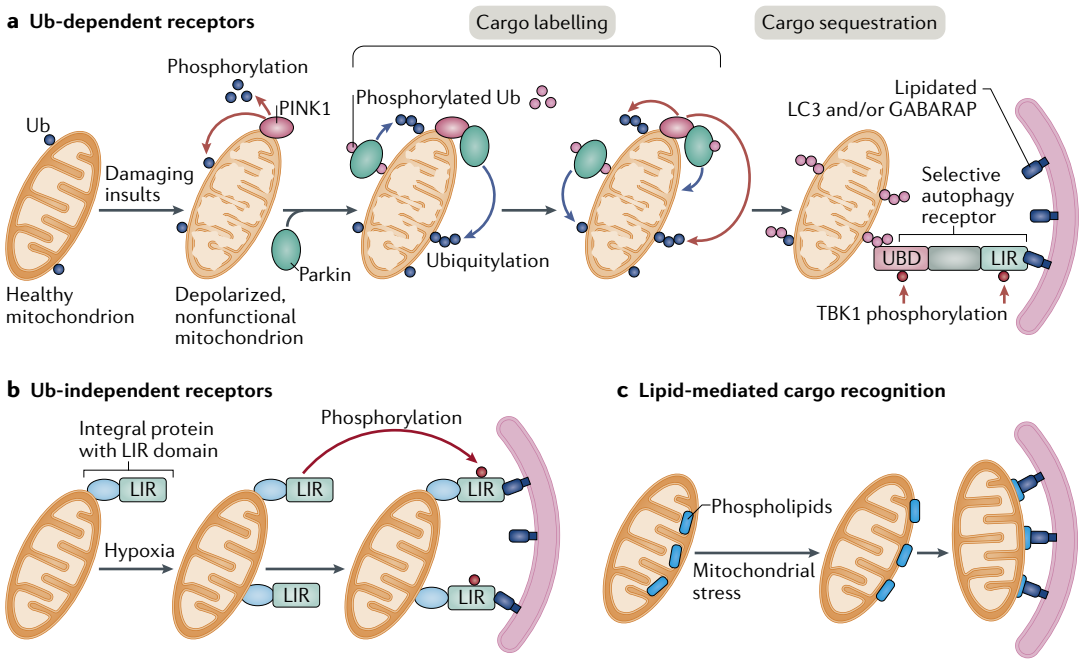
TSC2 (tuberous sclerosis 2) complex
Complex that is part of TSC that acts as a GTPase accelerating protein (GAP) for GTP-binding protein RHEB; because GDP-loaded RHEB is unable to activate mTORC1, TSC effectively shuts off mTORC1 signalling.

Raptor
Scaffold protein unique to mTORC1 (not present in mTORC2); binds substrates as well as regulators

Box 1 | **Cargo selection for selective autophagy**

Whereas starvation triggers bulk autophagy that nonspecifically engulfs any cytoplasmic material, certain signals or cellular events can evoke highly selective autophagic targeting of distinct cellular structures, such as damaged mitochondria (mitophagy), invading bacteria (xenophagy), aggregated proteins (aggrephagy) and others. Selective autophagy requires the labelling of cargo with 'eat-me' signals (most prominently ubiquitin (Ub) chains) recognized by autophagy receptors that link the cargo to the autophagic membrane via their light chain 3 (LC3)-interacting region (LIR). In selective autophagy, ULK1 is activated in an mTOR-independent manner that still awaits characterization. A recent report has implicated huntingtin (HTT), the protein product of the gene mutated in Huntington disease, as a possible molecular link between autophagic cargo and activation of ULK1[171]. In that study, HTT was shown to compete with mTOR complex 1 (mTORC1) for binding to ULK1, thus freeing ULK1 from mTORC1-mediated inhibition. HTT may also facilitate the interaction of the autophagy receptor sequestosome 1 (p62) with LC3 and K63-linked Ub chains, thereby coupling cargo recognition and activation of selective autophagy.

A well-studied targets of selective autophagy are mitochondria, which can be removed via different mechanisms depending on the physiological context. Upon damage or depolarization, the mitochondrial kinase PTEN-induced kinase 1 (PINK1) becomes stabilized and recruits the Ub E3 protein ligase Parkin (see figure part **a**). PINK1 and Parkin cooperate in a feedforward mechanism to assemble phosphorylated Ub (pUb) chains on several proteins of the outer mitochondrial membrane, which in turn recruit cargo receptors such as optineurin (OPTN), calcium-binding and coiled-coil domain-containing protein 2 (NDP52) and p62. In this process, PINK1 phosphorylates free Ub, polyUb attached by Parkin to the mitochondrial surface and the ubiquitin-like (UBl) domain of Parkin. These phosphorylation events enhance both the ubiquitin ligase activity of Parkin and its retention time on damaged mitochondria. Another player in mitophagy is TANK-binding kinase 1 (TBK1), which promotes coupling of the cargo to the phagophore by phosphorylating Ub-binding domains and LIRs of several cargo receptors, thereby increasing their affinity for pUb and LC3, respectively. Notably, mitophagy can also occur in a Ub-independent manner via mitochondrial proteins such as BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (NIX), FUN14 domain-containing protein 1 (FUNDC1) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), which possess an LC3-interacting region (LIR) and therefore function as direct cargo receptors (see figure part **b**). They are typically regulated by stress-dependent phosphorylation. Finally, lipids, including phospholipids, such as cardiolipin[172] and ceramide[173], have been shown to mediate mitophagy (see figure part **c**). In neuronal cells, cardiolipin is located at the inner membrane of healthy mitochondria, but upon mitochondrial damage, it is externalized and presented on the mitochondrial surface, where it is recognized by LC3.



GABARAP, γ-aminobutyric acid receptor-associated protein; UBD, ubiquitin D.

FOXO (forkhead box O) proteins
Family of transcription factors activated in response to cell stress; they regulate genes involved in cellular energy production, oxidative stress resistance, cell viability and proliferation.

Certain transcription regulators were implicated in the regulation of autophagy in different systems: the epigenetic reader bromodomain-containing protein 4 (BRD4) together with methyltransferase G9a were recently reported as repressors of a transcriptional programme of autophagic genes needed for autophagosome biogenesis[24], and the regulation of autophagy by FOXO (forkhead box O) proteins was demonstrated in cardiomyocytes[25,26].

Several other regulators of autophagic proteins have been described in recent studies. Beclin 1 is inhibited by antiapoptotic molecule BCL-2 (REF.[27]) and is also the target of several kinases: phosphorylation by ULK1, MAPKAPK (mitogen-activated protein kinase–activated protein kinase) 2 and 3, AMPK and DAPK (death-associated protein kinase) promotes autophagy, whereas AKT and EGFR (epidermal growth factor receptor) inhibit autophagy through Beclin 1 inactivation. PI3KC3–C1 is further

regulated by interaction with AMBRA1 (activating molecule in BECN1-regulated autophagy protein 1), which promotes autophagy[28], whereby ULK1-phosphorylated AMBRA1 is released from microtubules to allow Beclin 1 binding and consequent PI3KC3–C1 activation[29]. In chondrocytes, fibroblast growth factor 18 (FGF18) and its receptor FGFR4 activate the VPS34–Beclin 1 complex in a JNK-dependent manner to initiate autophagy[30]. Progestin and adipoQ receptor family member 3 (PAQR3), a Golgi complex-localized multipass transmembrane protein, was found to shift the balance towards PI3KC3 association with ATG14 instead of with UVRAG upon glucose starvation and thereby increase autophagy[31]. Finally, it has been recently reported that autophagy in livers of fasting mice is regulated by acetylation of VPS34, which is mediated by the histone acetyltransferase p300 (REF.[32]).

The mechanistic determinant for recruitment of ULK1 to the PAS is largely unclear. In a recent study, the Golgi-localized WW domain-containing adaptor with coiled coil (WAC) was identified as a positive regulator of autophagy[33]. In a subsequent study, WAC was found to mediate translocation of GABARAP — a factor that mediates phagophore expansion — from the Golgi complex to the centrosome[34]. It was proposed that centrosomal GABARAP is then trafficked (possibly via microtubules) to the phagophore, where it recruits and activates the ULK1 complex. This centrosomal pool of GABARAP might support sustained activation of the ULK1 complex during autophagosome formation. PI3KC3–C1 is targeted to the PAS by ATG14 (REFS[35,36]) possibly through phosphorylation by ULK1 and consequent interaction with ATG13[37], while ATG14 is regulated by interaction with type Iγ PI-phosphate 5-kinase (PIPKIγi5), an enzyme that generates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)[38]. Recruitment of ATG9 is regulated by the ULK1 complex[10,39] and by transport protein particle complex III (TRAPPIII), an activator of the ER-to-Golgi complex trafficking factor Ras-related protein RAB1 (also known as RAB1A)[40,41]. Moreover, guanine nucleotide exchange C9ORF72, a protein that is mutated in patients with amyotrophic lateral sclerosis (ALS) or with frontotemporal dementia, was recently shown to interact with ULK1 and RAB1 (REF.[42]), suggesting that RAB1 coordinates ATG9 recruitment with the activity of ULK1.

Of note, phagophore nucleation (and possibly expansion) probably also involves actin scaffolding, as autophagosome formation is promoted by F-actin-capping protein CapZ[43] and WASP homologue-associated protein with actin, membranes and microtubules (WHAMM) recruited to the PAS by PI3P and by actin nucleation-promoting factor junction-mediating and -regulatory protein (JMY), which is targeted to the phagophore via its LC3-interacting region (LIR; see also below)[44].

Phagophore expansion. The ATGs most prominently implicated in phagophore expansion are the Ub-like ATG8 family members[45]. Nascent pro-ATG8s are processed at their C-termini by the cysteine protease ATG4, exposing a glycine residue that is essential for their conjugation to PE[45]. The specificity of the four distinct ATG4 isoforms is not fully characterized, but ATG4B has been shown to recognize all ATG8s, whereas ATG4A is more specific to GABARAPs[46,47]. The processed ATG8s are activated by the E1-like enzyme ATG7 and conjugated to membrane-associated PE by the activity of ATG3, thereby converting it from a freely diffuse form (for LC3 this form is known as LC3-I)into a membrane-anchored, lipidated form (for LC3 referred to as LC3-II)[8]. For efficient PE conjugation in vivo, ATG3 requires stimulation by E3-like activity of the ATG12~ATG5 conjugate, formed by activation of ATG12 by ATG7 and conjugation to ATG5 by E2-like ATG10 (REF.[8]). The activity of ATG12~ATG5 is localized to the PAS by interaction with ATG16L in a dimeric ATG12~ATG5–ATG16L complex[48,49] that is recruited to the PAS through interaction of ATG16L1 with WIPI2 (REF.[50]). ATG16L1 can form homo-oligomers through a coiled-coil domain, which may allow ATG16L1 to crosslink multiple ATG12~ATG5 conjugates into a single large protein complex that possibly serves to scaffold the phagophore[51]. Conjugation of ATG8s to PE promotes phagophore expansion (and possibly also sealing)[52]. This conjugation event is suggested to occur on ER exit sites following starvation-induced and FIP200-mediated relocation of ER exit factor prolactin regulatory element-binding protein (SEC12) to the ER–Golgi intermediate compartment (ERGIC)[53,54] — in line with the observations that phagophores form in apposition to ER exit sites[55]. Notably, this view has been recently challenged by studies indicating that autophagosomes can form without the conjugation machinery[56] or even in the absence of all ATG8s[57]. Aside from their contribution to phagophore expansion, phagophore-anchored ATG8s also facilitate cargo recruitment in selective autophagy, as they interact with LIRs of cargo receptors (which themselves recognize the cargo through 'eat-me' signals, such as Ub or galectins (BOX 1)).

Apart from C-terminal processing of nascent ATG8s, ATG4 is also capable of deconjugating ATG8s from PE to release it from the membrane and limit phagophore expansion. Both activities of ATG4 are required for the normal progression of autophagy. As the autophagic activities of ATG8s are attributed to their conjugation to PE, it was originally postulated that ATG4 deconjugating activity must be tightly regulated both in time and in space[58]. Accordingly, in order to function properly on the autophagic membrane, lipidated ATG8s should be protected from ATG4 (REFS[58–60]). This may be regulated by mitochondria-generated reactive oxygen species (ROS)[58], in line with the suggestion that phagophores form preferentially at ER–mitochondria contact sites. Alternatively, ATG8s might be protected from deconjugation by inhibition of ATG4 through phosphorylation by ULK1 (REFS[59,60]).

Targeting of ATG8s to autophagic membranes can also be regulated by additional post-translational modifications. For example, phosphorylation of LC3 by protein kinase A (PKA) negatively regulates its autophagic activity[61]. Finally, autophagosomal size is also controlled by the AMPK-related kinases NUAK family SNF1-like kinase 2 (NUAK2) and

JNK
Member of the MAPK family activated by extracellular signals; associated with several pathological conditions, including neurodegenerative diseases, inflammation and cancer.

E1
Ubiquitin (Ub)-activating enzyme; first enzyme in the E1–E2–E3 ubiquitylation cascade that activates Ub in an ATP-dependent manner.

E3
Ubiquitin (Ub)-ligating enzyme; cooperates with E2 to attach Ub to a lysine residue in the target protein. Only component of the Ub machinery that interacts with the target, thus conferring substrate specificity to the reaction.

E2
Ubiquitin (Ub)-conjugating enzyme; takes over activated Ub from E1 and hands it over to E3. Plays a key role in defining the linkage type of Ub conjugation when chains of multiple Ub molecules are assembled.

ER exit sites
Areas of the endoplasmic reticulum (ER) where transport vesicles that contain lipids and proteins made in the ER detach from the ER and move to the Golgi complex.

Galectins
Carbohydrate-binding lectins that recognize intracellular bacteria-containing vesicles when their membrane integrity is compromised.

serine/threonine-protein kinase BRSK2 through ATG2 and WIPI4 (REF.[22]). Accordingly, WIPI molecules function as PtdIns3P effectors at the nascent autophagosome, acting as scaffold molecules with distinct interactions to different autophagic factors[50]. WIPI4 interacts with ATG2 to regulate autophagosome formation by an as yet unclear mechanism[22].

Autophagosome maturation. Following expansion and sealing of the phagophore, the autophagosome undergoes maturation, which involves gradual clearance of ATGs from the nascent autophagosome outer membrane and recruitment of machinery responsible for lysosomal delivery (microtubule-based kinesin motors) and machinery that mediates fusion with the lysosome, encompassing SNAREs: syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29), on the autophagosome and vesicle-associated membrane protein 8 (VAMP8), on the lysosome[62,63], and the homotypic fusion and protein sorting (HOPS) complex, which mediates membrane tethering to support SNARE-mediated fusion. These processes all occur in a poorly characterized and probably coordinated manner that is slowly emerging[64] (see also REF.[65] for review).

ATG8s drive maturation by linking the autophagosome to kinesins through autophagy-specific kinesin adaptors such as FYCO1 (FYVE and coiled-coil domain-containing protein 1)[66]. ATG8s also recruit — via pleckstrin homology domain-containing family M member 1 (PLEKHM1) — the HOPS complex to the autophagosome[67]. Recruitment of the HOPS complex to the autophagosome was also proposed to be mediated by UVRAG, which is negatively regulated by mTORC1 (REF.[68]), thus potentially broadening the range of mTORC1 activities to late events along the autophagic process. However, a later study suggested an indirect role for UVRAG in autophagy that is secondary to its role in late stages of endocytic degradation[69].

There is now evidence that post-translational modifications of ATG8s further regulate autophagosome maturation, as the phosphorylation of LC3 on residue Thr50 by the Ste20 Hippo kinase orthologues serine/threonine-protein kinase 3 (STK3) and STK4 was recently found to be essential for autophagosome–lysosome fusion and for clearance of intracellular bacteria by autophagy[70]. Interestingly, the phagophore nucleation factor ATG14 was recently implicated in autophagosome maturation as well. ATG14 was shown to be recruited to the autophagosomal outer membrane by interaction with STX17 and to promote membrane tethering to enhance SNARE-mediated fusion[63].

Medical implications

Extensive research over the past two decades has not only established a central role for autophagy in cellular homeostasis but also unravelled molecular links to various disease conditions (TABLE 2). Chemical or genetic disturbance of autophagy and the age-dependent decline in autophagic activity have been implicated in the progression of cancer, neurodegeneration and immune diseases, as well as ageing [71].

Complex roles of autophagy in cancer development and progression. Autophagy is an important process during cancer progression, but the exact roles of autophagy in cancer cells are strongly context-dependent (FIG. 2a). Its cytoprotective function is believed to have tumour-suppressive potential before the onset of tumorigenesis, and loss of autophagy has been associated with increased risk of cancer[72]. However, autophagy has also been shown to allow premalignant cells to escape genotoxic stress and inflammation that promote tumorigenesis. There is good evidence, moreover, that autophagy provides cancer cells with metabolic plasticity, allowing them to thrive in suboptimal environments[73] and to exploit the prosurvival activity of autophagy to cope with therapy-induced stresses[74–76]. Accordingly, many types of advanced cancers exhibit high autophagic activity[77], and it was proposed that certain tumours, such as pancreatic cancer[78–80] or cancers with mutant RAS (rat sarcoma) genes[81], are highly dependent on autophagy. Interestingly, it has been revealed that autophagy induction is a side effect of many cancer therapies[82], and thus, pharmacological inhibition of autophagy has been proposed as a valid strategy to enhance the efficacy of therapies and to avoid resistance to treatment in certain cancers[81,83,84] (TABLE 2). Notably, some reports also highlight a beneficial role for autophagy activation in cancer therapies involving the induction of immunogenic cell death. In this context, autophagy-competent dying tumour cells actively release ATP[85–87] and the high-mobility group box 1 protein B1 (HMGB1)[88,89], which recruit immune effectors into the tumour bed to trigger a tumour-specific immune response. Thus, activation of autophagy rather than its inhibition could be considered as a strategy to boost the efficacy of cancer therapy[83]. In accordance with this notion, caloric restriction (which promotes autophagy by inactivation of mTORC1) was found to enhance tumour immunosurveillance but had this effect only in the case of autophagy-proficient tumours[90]. In order to therapeutically exploit these findings, it will be necessary to identify chemotherapy and/or radiotherapy regimens that trigger an optimal tumour-targeting immune response as well as to define the types of cancer that are sensitive to this treatment strategy. The genetic context was also shown to be important for determining the role of autophagy in cancer. For example, in a mouse model of pancreatic ductal adenocarcinoma, the loss of autophagy prevents the formation of high-grade pancreatic intraepithelial neoplasias in the presence of p53, whereas in the absence of p53, autophagy inhibition accelerates tumour growth[91]. Thus, autophagy seems to be a double-edged sword in the context of cancer therapies, and it remains to be established whether it can be successfully targeted — inhibited or induced — for therapeutic benefit.

The emerging notion that autophagy can shape the tumour microenvironment is further corroborated by the fact that autophagy can facilitate polarized sorting and unconventional secretion of certain cytosolic proteins[92,93]. Indeed, oncogenic RAS-driven invasion was shown to be dependent on autophagy-mediated secretion of multiple factors, including the pro-migratory cytokine interleukin 6 (IL-6) and WNT5a, which are

SNAREs
Proteins that mediate the fusion of vesicles with target membranes. SNARE proteins on the vesicle (v-SNAREs) and on the target membrane (t-SNAREs) combine to form a *trans*-SNARE complex that provides the force for membrane fusion.

Hippo kinase
A kinase that functions as a central node in the regulation of cell division and controls organ size in flies and mammals as well as the growth of cancer cells.

High-mobility group box 1 protein (HMGB1)
A protein that senses and coordinates the cellular stress response acting as a DNA chaperone, autophagy sustainer and protector from apoptotic cell death. Outside the cell, it functions as a prototypic damage associated molecular pattern molecule (DAMP).

Unconventional secretion
Comprises the translocation across the plasma membrane of cargo without a signal peptide or a transmembrane domain and cargos that reach the plasma membrane by bypassing the Golgi apparatus despite entering the endoplasmic reticulum (ER).

Table 2 | **Human diseases linked to autophagy and clinical translation**

Disease	Mechanism	Compounds
Autophagy activation in neurodegenerative diseases		
Alzheimer disease	mTOR inhibition (via 5-HT_6R activation)	AVN-211; Lu AE58054 (idalopirdine); SB-742457
	Inhibition of AKT–mTOR pathway	rAAV/Aβ vaccine
	ACAT1 inhibition	F12511
	mTOR inhibition	Rapamycin, latrepirdine and metformin
	AMPK activation	Resveratrol and resveratrol-like small molecules
	Lysosomal acidification	Nicotinamide
	GSK3β and IMPase inhibition	Lithium
	Unclear mechanism	Berberine
	MTMR14 (autophagy inhibitor) inhibition	AUTEN-67
Parkinson disease	NRF2 activation	DMF
	TFEB activation	Curcumin analogue
	Beclin 1 complex activation	*BECN1* gene transfer
	TFEB regulation	*TFEB* gene
	Beclin 1 activation	Dual GLP-1–GIP receptor agonists
ALS	Unclear mechanism	Berberine
Huntington disease	MTMR14 inhibition	AUTEN-67
	mTOR inhibition	Rapamycin
	Unclear mechanism	Berberine
	Calpain inhibition	Calpastatin
	Unknown	Rilmenidine
	Unknown	Trehalose
	mTOR activation	Constitutively active *RHEB* gene product
Interventions involving autophagy inhibition in cancer		
Breast cancer	Autophagy inhibition + microtubule inhibition	CQ + taxols
	Autophagy inhibition	CQ
Prostate cancer	Autophagy inhibition + BCL-2 inhibitor + antiandrogen	HCQ + ABT-263 + abiraterone
	Autophagy inhibition	HCQ
	Autophagy inhibition + androgen receptor inhibition	Metformin hydrochloride + enzalutamide
Pancreatic cancer	Autophagy inhibition + inhibition of DNA synthesis	CQ + gemcitabine
	Autophagy inhibition + inhibition of DNA synthesis + microtubule inhibition	HCQ + gemcitabine + abraxane
	Autophagy inhibition + inhibition of DNA synthesis	HCQ + gemcitabine
	Autophagy inhibition	CQ
Small-cell lung cancer	Autophagy inhibition	CQ
	Autophagy inhibition + DNA damage	CQ + radiotherapy
Non-small-cell lung cancer	Autophagy inhibition + microtubule inhibition + DNA damage + inhibition of angiogenesis	HCQ + paclitaxel + carboplatin + bevacizumab
Melanoma	Autophagy inhibition + DNA damage + DNA repair inhibitor	CQ + radiation + DT01
	Autophagy inhibition + MEK inhibition	HCQ + trametinib
	AKT–mTOR signalling	Curcumin
Colorectal cancer	Autophagy inhibition + alkylation + DNA damage + inhibition of angiogenesis	HCQ + oxaliplatin + 5-FU + bevacizumab
	Autophagy inhibition + inhibition of angiogenesis + alkylation and antimetabolite	HCQ + bevacizumab + XELOX
	Autophagy inhibition + HDAC inhibitor	HCQ + vorinostat
Renal cell carcinoma	Autophagy inhibition + mTOR inhibitor	HCQ + RAD001

Table 2 (cont.) | **Human diseases linked to autophagy and clinical translation**

Disease	Mechanism	Compounds
Interventions involving autophagy inhibition in cancer (cont.)		
Solid tumours	Autophagy inhibition + HDAC inhibitor	HCQ + vorinostat
	Autophagy inhibition + DNA damage	CQ + carboplatin and/or gemcitabine
Multiple myeloma	Autophagy inhibition + proteasome inhibition + alkylation	CQ + velcade + vyclophosphamide
Glioblastoma	Autophagy inhibition + DNA damage and/or alkylation	CQ + chemoradiation with temozolomide
Interventions involving autophagy activation in cancer and cancer-related phenotypes		
Adenocarcinoma bone metastasis	p53-dependent autophagy induction	Fluvastatin (HMG-CoA reductase inhibitor)
Hepatocellular carcinoma	AMPK activation	Palbociclib
Inclusion body myositis	mTOR inhibition	Rapamycin
Desmoid-type fibromatosis	mTOR inhibition	Rapamycin
Advanced cancers	mTOR inhibition + HDAC6 inhibition + autophagy inhibition	Rapamycin + vorinostat + HCQ
Other interventions		
Infection	AMPK-mediated autophagy activation	Ohmyungsamycins
	Autophagy activation by mTOR inhibition	Statin, gefitinib and carbamazepine
	Autophagy activation	TAT-Beclin1
Diabetes	SIRT1 upregulation	Resveratrol

For references, see Supplementary Table 1. 5-FU, 5-fluorouracil; 5-HT_6R, 5-hydroxytryptamine 6 receptor; ACAT1, acyl-CoA:cholesterol acyltransferase 1; ALS, amyotrophic lateral sclerosis; AMPK, 5′ AMP-activated protein kinase; BECN1, Beclin 1; CQ, chloroquine; DMF, dimethyl fumarate; DT01, DNA repair inhibitor; GLP-1/GIP, glucagon-like peptide 1/glucose-dependent insulinotropic polypeptide; GSK3β, glycogen synthase kinase-3β; HCQ, hydroxychloroquine; HDAC, histone deacetylase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IMPase, inositol monophosphatase; MTMR14, myotubularin-related protein 14; NRF2, nuclear factor erythroid 2-related factor 2; RAD001, 40-*O*-(2-hydroxyethyl) derivative of sirolimus; rAAV/Aβ, recombinant adeno-associated viral vector/amyloid-b; SIRT1, NAD-dependent protein deacetylase sirtuin-1; TAT, transactivator of transcription peptide derived from a region of Beclin 1, which binds HIV-1 Nef; TFEB, transcription factor EB.

normally secreted via the conventional pathway[94]. In addition, recent findings point to a close relationship between autophagy and the biogenesis and secretion of exosomes[95,96]. Exosomes transfer lipids, proteins, mRNAs, non-coding RNAs and even DNA out of cells and have been shown to promote tumour growth, alter the tumour microenvironment, facilitate cancer cell dissemination, modulate immune responses and mediate resistance to therapy[97].

To acquire metastatic potential, adherent cancer cells need to gain motility, which is achieved through epithelial–mesenchymal transition (EMT). Intriguingly, signals that trigger EMT, such as hypoxia or transforming growth factor-β (TGFβ), also activate autophagy pathways[98]. Yet, as activation of autophagy was shown to cause downregulation of major transcription factors of the EMT process, autophagy seems to inhibit rather than support EMT in most types of cancer[99–101]. Moreover, cadherin 6, which specifically marks cells undergoing EMT and actively drives the EMT process, downregulates autophagy by directly interacting with and blocking the functions of several autophagic proteins[102], suggesting that autophagy activation is not favoured during EMT itself.

A feature closely linked to EMT is anoikis, a specific form of apoptotic cell death that results from the prolonged detachment of cells from the extracellular matrix (ECM) and is mediated by BCL-2 protein family members, including Bcl-2 modifying factor (BMF) and Bcl-2-like protein 11 extra-long isoform (BIM-EL). Anoikis represents a critical challenge to metastasizing tumour cells. However, autophagy activated upon loss of ECM–integrin receptor engagement can promote resistance to anoikis in several tumour models, possibly by alleviating metabolic deficiencies in ECM-detached cells[98,103,104]. Intracellular signals governing this process remain poorly defined but might involve the integration of multiple pathways, including those that activate autophagy upon accumulation of ROS[105], glucose starvation, ER stress signalling via the PERK pathway[106,107] and activation of the IKK (IκB kinase) complex[108], which is a central activator of the NF-κB (nuclear factor-κB) pathway. Moreover, emerging evidence points to direct, negative regulation of the autophagy factor Beclin 1 by the proapoptotic factors BIM-EL and BMF: the interaction of BIM-EL with Beclin 1 was shown to inhibit autophagosome formation by sequestering Beclin 1 to microtubules, whereas BMF was reported to stabilize the inhibitory Beclin 1–BCL-2 protein complex[109–111]. However, the exact mechanisms and the pathophysiological consequences of this crosstalk between autophagy and anoikis are not yet fully understood.

Besides preventing anoikis, the ECM contact also has a major role in cancer cell migration. Cells bind to the ECM through large protein complexes called focal adhesions. In order for cells to migrate, their focal adhesions need to be taken apart and then reconstructed in a coordinated fashion. Autophagy contributes to focal adhesion remodelling by controlling the turnover of key components of focal adhesions, including paxillin, vinculin, zyxin and

Exosomes
Small extracellular vesicles that contain various molecular constituents and are released directly from the plasma membrane or when multivesicular bodies fuse with the plasma membrane.

NF-κB (nuclear factor-κB) pathway
A transcription factor that controls cytokine production and cell survival and plays a key role in the cellular response to infection. Disturbance of the pathway has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development.

a Autophagy and cancer

Cancer progression		Role of autophagy
Cancer initiation	Antitumoral	Protection against stress (metabolic, oxidative, inflammatory)
Growth of primary tumour	Protumoral	Protection against stress (metabolic, oxidative, inflammatory)
EMT	Antitumoral	Downregulation of EMT-promoting transcription factors
Anoikis resistance	Protumoral	Unclear mechanism, multiple pathways involved
Migration	Antitumoral	RHOA degradation
	Protumoral	Focal adhesion turnover

Cancer treatment		Role of autophagy
Treatment resistance	Protumoral	Cytoprotection
Immunogenic cell death	Antitumoral	Secretion of factors that trigger tumour-specific immune response

b Autophagy in cell migration

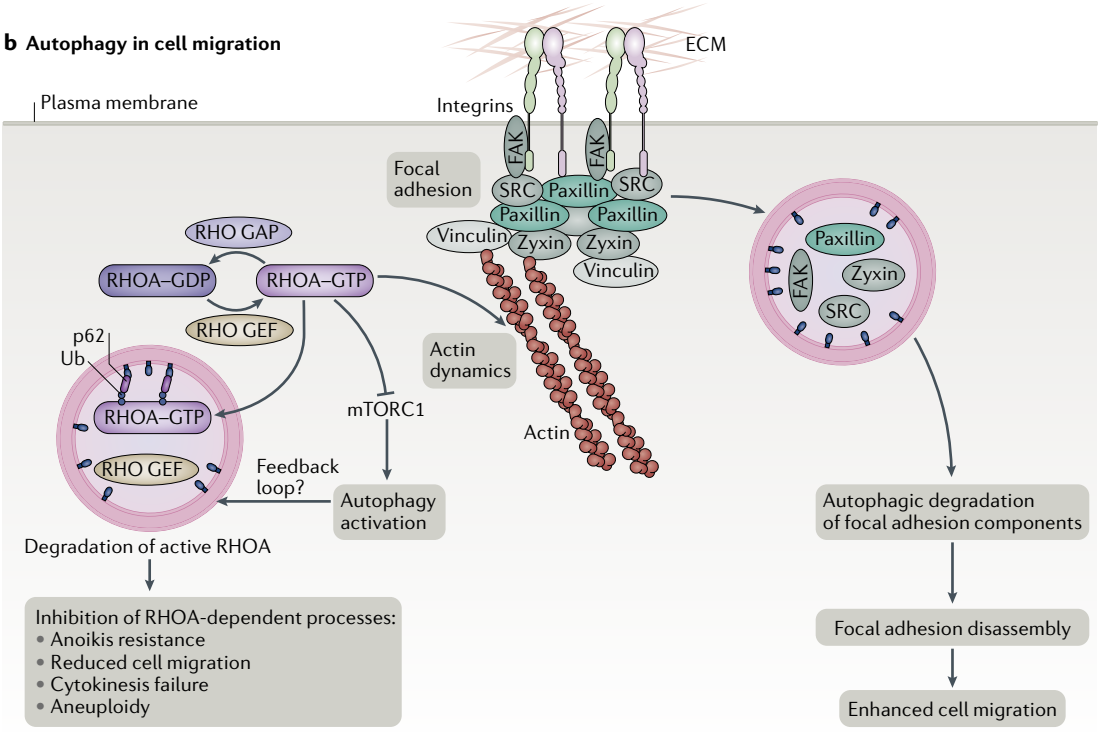


Fig. 2 | **Autophagy in cancer. a** | Autophagy impacts several aspects of cancer progression. High autophagic activity is believed to be cytoprotective and to suppress cancer initiation. However, in the primary tumours (after successful initiation), autophagy is often upregulated to overcome stresses resulting from fast growth (such as protein stress) as well as low nutrient availability (starvation) inside the tumour mass. Other cellular processes are associated with cancer progression and spreading crosstalk with the autophagy pathway. The upregulation of autophagy during epithelial–mesenchymal transition (EMT) appears to have an inhibitory effect on EMT, as several EMT-promoting transcription factors are downregulated in an autophagy-dependent manner. In contrast, another process that is required for cancer cells to gain migratory capacity and spread, anoikis resistance, is promoted by upregulation of autophagy in many cancers, yet the underlying mechanisms are unclear. Cell migration can be both promoted by autophagy (turnover of focal adhesions) and inhibited (autophagic degradation of actin dynamics regulator transforming protein RHOA); see also part **b**. On one hand, cancer therapies can induce autophagy, which contributes to the development of resistance. On the other hand, autophagy was reported to be required for immunogenic cancer cell death and was suggested to be antitumorigenic. **b** | Autophagy regulates cell migration in at least two opposing ways: on one hand, autophagy directly degrades active (GTP-bound) RHOA and the RHOA guanine nucleotide exchange factor (GEF) H1 in a ubiquitylation-dependent manner mediated by recognition through autophagy receptor sequestosome 1 (p62). This impacts actin dynamics, thus inhibiting cell migration and other RHOA-dependent cellular processes. Interestingly, autophagic RHOA degradation also contributes to anoikis resistance. Intriguingly, RHOA has been shown to inhibit signalling upstream of mTORC1, thus stimulating autophagy in a potential negative feedback loop. On the other hand, autophagy mediates the disassembly of focal adhesions by degrading several focal adhesion components, thus contributing to increased cell migration. ECM, extracellular matrix; FAK, focal adhesion kinase; mTORC1, mTOR complex 1; Ub, ubiquitin.

Leading edge
Front edge of a cell that is pushed forward by rapid actin polymerization.

focal adhesion kinase (FAK), and loss of autophagy inhibits migration and focal adhesion turnover at the leading edge[112,113] (FIG. 2b). Moreover, autophagy has been shown to directly target and degrade active transforming protein RHOA[114] as well as the RHOA–guanine nucleotide exchange factor (GEF) H1 (REF.[115]), which are crucial regulators of actin dynamics and cell migration. Remarkably, RHOA has also been implicated in mediating anoikis through cytoskeletal tension-dependent cell death in unattached cells[116]. Thus, RHOA degradation could be one way by which autophagy contributes to anoikis resistance. Importantly, in other contexts, autophagy-mediated RHOA degradation may inhibit cell migration as well as other RHOA-dependent events, including cell division, leading to cytokinesis failure or aneuploidy[114]. Intriguingly, RHOA can repress signalling through mTORC1, thus enhancing autophagy[117], further highlighting the complex interplay between cell–ECM attachment, cell migration and autophagy.

Taken together, autophagy can both suppress and promote cancer progression and metastasis at several stages. This complicates therapeutic intervention (BOX 2) and makes it necessary to evaluate the type of tumour cell, its genetic background, the stage of tumour progression and the tumour microenvironment in order to achieve the desired effect of autophagy modulation and avoid potential aggravation of the disease.

Autophagy against neurodegenerative diseases. Among the hallmarks of neurodegenerative diseases (including Alzheimer disease, Parkinson disease, Huntington disease, ALS, Vici[118], hereditary spastic paraplegia[119], static encephalopathy of childhood with neurodegeneration in adulthood (SENDA)[120] and others) are aggregates of misfolded or unfolded proteins that accumulate inside neuronal cells, eventually causing severe disturbances in their function and/or their death (FIG. 3).

In healthy cells, proteins that are not properly folded are tagged with Ub and degraded by the proteasome[121]. However, proteasomal activity is prone to impairment by various internal and external stresses and declines with age. If the degradative capacity of the proteasome is overloaded, the autophagy system becomes activated to remove accumulating aggregates as well as organelles that are irreparably damaged by aggregated and non-functional proteins[121]. Indeed, the most common cause of death of autophagy-deficient animals is neurodegeneration accompanied by the accumulation of ubiquitylated protein aggregates[122,123]. Moreover, numerous proteins that are mutated in neurodegenerative diseases have been implicated in autophagy[124,125] or in lysosomal function[126], and transcriptome studies using samples from patients revealed alterations in autophagy-related signalling[127–129]. The autophagic cargo receptor sequestosome 1 (p62), which binds Ub, has a key role in the clearance of protein aggregates, and post-mortem analysis of p62-positive inclusions is a defining diagnostic

Box 2 | Autophagy as a pharmacological target

The first US Food and Drug Administration (FDA)-approved agent capable of inhibiting autophagy was chloroquine, a drug previously used to treat malaria and arthritis, which also blocks autophagy by disrupting lysosome acidification[174]. Now, multiple targets within the pathway have been or are being evaluated for pharmacological intervention of autophagy (TABLE 2), including mTOR, serine/threonine protein kinases ULK1 and ULK2[175,176], vacuolar protein sorting 34 (VPS34)[177–179], and interactions within the Beclin 1 complex[180], the E1-like enzyme autophagy-related protein 7 (ATG7)[181] and ATG4B — the protease that processes pro-LC3 (light chain 3)[182].

The most thoroughly tested inducer of autophagy is rapamycin, which inhibits mTOR complex 1 (mTORC1) in mouse and fly models of various neurodegenerative diseases. However, considerable side effects on cellular pathways other than autophagy precluded its therapeutic use in humans[183]. Additionally, natural (often dietary) compounds, including resveratrol, polyphenols, berberine, artemisinin, sesamol, trehalose or spermidine, have moved into the focus of pharmacologists, yet knowledge of the mechanisms of action and potential side effects of these substances is currently incomplete[184–192]. Notably, in addition to pharmacological interventions, caloric restriction and exercise were also shown to induce autophagy and to contribute to protection against diabetes in mice[193], and the effect of alternate-day fasting on human metabolism and autophagy is currently being tested in a phase I clinical trial (NCT02673515).

In most cancer therapies, inhibition of autophagy is combined with other therapeutic interventions, including radiation, chemotherapies and targeted agents, including DNA-damaging agents, histone deacetylase (HDAC) inhibitors, proteasome inhibitors, mitotic inhibitors, antiandrogens and kinase inhibitors[194,195]. Unfortunately, although some reports indicated that autophagy inhibition might increase chemosensitization and may overcome acquired resistance to other anticancer agents, multiple clinical trials testing the efficacy of autophagy inhibition in cancer patients have been largely disappointing and have served to underline the vast complexity of networks in which autophagy is embedded. As the crosstalk between autophagy pathways and other cellular systems is usually reciprocal, modulation of autophagy activity not only affects the efficacy of protein aggregate clearance or the elimination of damaged organelles but also likely impacts the magnitude or duration of other fundamental cellular pathways, such as NF-kB signalling, cell migration or cell death programmes. Finally, autophagy regulation in the tumour stroma and in tumour cells may differ: whereas inhibition of autophagy in tumour cells might trigger cell death, it could at the same time promote the release of survival factors in the tumour stroma (particularly fibroblasts and tumour-infiltrating immune cells)[196], thereby precluding a positive therapeutic outcome. Thus, decisions as to whether autophagy activity in a certain disease condition, particularly in cancer, should be upregulated or downregulated are not trivial and require careful evaluation of tumour type, stage and microenvironment.

Whereas targeting of autophagy in cancer turns out to be a delicate task, the consensus with respect to neurodegeneration is that autophagy activation protects against several neurodegenerative disorders[197,198]. Nevertheless, also in the context of neurodegeneration, therapeutic targeting of autophagy is challenging, and optimal dosage of inhibitors and timing of inhibition are crucial parameters for maximal therapeutic efficiency[199]. Indeed, as with cancer therapies, the specific targeting of autophagy without affecting other cellular processes is currently one of the major challenges in the field.

Lastly, several FDA-approved drugs with proautophagy activity (see TABLE 2) have been shown to limit infection and inflammation in mouse models[200,201], and the peptide TAT-Beclin1 improved the outcome of chikungunya and West Nile virus infections in mice[180]. However, evaluations of human patients through clinical trials are still unavailable.

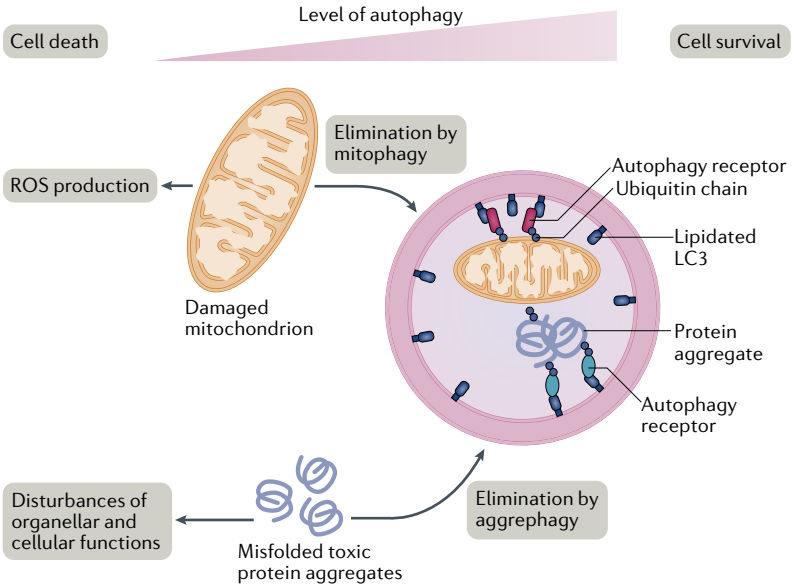


Fig. 3 | **Autophagy in neurodegeneration.** Autophagy protects against neurodegeneration by eliminating two hallmarks of neurodegenerative diseases: defective mitochondria and toxic protein aggregates. Damaged mitochondria produce high levels of reactive oxygen species (ROS) that pose a threat to many cellular components, including proteins, lipids and DNA. Protein aggregates, which are exacerbated by ROS-mediated oxidative damage, compromise the function of organelles and are considered particularly toxic for neurons. Reduced autophagy activity (age-related, pharmacologically or genetically caused) therefore increases the risk of neurodegenerative diseases. Accordingly, pharmacological stimulation of autophagy could be an effective therapeutic strategy against neurodegenerative diseases. LC3, light chain 3.

marker in several neurodegenerative diseases[130]. p62 participates in both aggregate formation by targeting misfolded aggregated proteins to the aggresome (a single intracellular location in which misfolded proteins are sequestered to minimize potential cytotoxic effects)[131] and the subsequent sequestration of aggresomes by the phagophore[132,133].

Besides potentially toxic protein aggregates, dysfunctional mitochondria have also been identified as a major cause of neurodegeneration. They pose a considerable threat to cells because they elevate cellular ROS levels that might in turn damage both the proteome and the genome (FIG. 3). Therefore, to maintain mitochondrial homeostasis, cells separate damaged mitochondria from the mitochondrial network and remove them by selective autophagy (termed mitophagy) (see BOX 1). Mitophagy is predominantly regulated by the PINK1 (PTEN-induced kinase 1)–Parkin pathway, which is activated upon depolarization of the mitochondrial membrane potential and involves a sophisticated interplay of PINK1-mediated phosphorylation and Parkin-mediated ubiquitylation events on the outer mitochondrial membrane, resulting in recruitment of autophagic machinery and the selective sequestration of ubiquitylated mitochondria within autophagosomes[134–136]. Mutations in Parkin and PINK1 are strongly associated with early-onset Parkinson disease[137]. In addition to the PINK1–Parkin pathway, NIP-like protein X (NIX; also known as BNIP3L) can serve as an alternative mediator of mitophagy in neurons. Recent evidence suggests that NIX overexpression restores mitophagy and mitochondrial function in Parkin-deficient or PINK1-deficient cell lines derived from patients with Parkinson disease[138].

A number of studies have also uncovered a link between TANK-binding kinase 1 (TBK1) and ALS[139–142] as well as Parkinson disease[143]. TBK1 belongs to the IKK family of kinases involved in innate immunity signalling pathways, but it also has a major role in autophagy and mitophagy. Through inducible phosphorylation of ubiquitylated cargo receptors (which, in addition to p62, include OPTN (optineurin) and NDP52 (calcium-binding and coiled-coil domain-containing protein 2; also known as CALCOCO2)), TBK1 enhances their affinity to Ub on the cargo, LC3 on the autophagosome or both, thereby contributing to efficient recruitment of ubiquitylated cargo to autophagosomes[136,144,145].

Taken together, in contrast to cancer where the function of autophagy is highly context dependent, activation of autophagy is clearly beneficial for counteracting the mechanisms involved in neurodegenerative diseases, and currently, autophagy induction is being explored as a strategy for neurodegenerative disease prevention as well as for the treatment of advanced-stage disease (BOX 2; TABLE 2).

Autophagy in infection, inflammation and immunity. Autophagy has been implicated in a variety of immune functions, such as removal of intracellular bacteria[146–148], inflammatory cytokine secretion[149], control of inflammation, antigen presentation[150,151] and lymphocyte development[152]. The importance of autophagy for these functions is highlighted by the susceptibility of autophagy-deficient animals to infection and the implication of autophagy defects in autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, diabetes and multiple sclerosis[153,154]. Autophagy also acts within tumour cells to modulate recruitment of and interaction with components of both the adaptive and innate immune systems[155].

Mechanistically, autophagy extensively crosstalks with inflammatory signalling cascades, including multiple context-specific and bidirectional interactions with the IKK–NF-κB pathway[156,157]. NF-κB can induce autophagy by transactivating Beclin 1 (REF.[158]). Moreover, in the presence of various physiological and pharmacological stress signals, the IKK complex can induce autophagy[156]. Yet, the NF-κB pathway may also inhibit autophagy, for example, in the context of tumour necrosis factor-α (TNFα)-induced cell death[159] and in macrophages infected by *Escherichia coli*[160]. Reciprocally, in several cell lines, TNFα-driven NF-κB activation requires a functional autophagy pathway[161]. Autophagy can also suppress NF-kB signalling by the autophagic degradation of active IKKβ, mediated either by KEAP1 (Kelch-like ECH-associated protein 1)[162] or by the E3 ubiquitin-protein ligase RO52 (also known as TRIM21)[163].

In a process called xenophagy, autophagy also directly targets and eliminates invading bacteria such

as *Mycobacteria*[164], *Listeria*, *Salmonella*, *Legionella*, *Shigella*, *Listeria* and group A streptococcus (see REF.[147] for review). As soon as these bacteria enter the cytosol, they are labelled with various types of Ub chain and galectin and sequestered by autophagic membranes involving the same autophagic receptors (p62, NDP52 and others) that also engage endogenous selective autophagy substrates. The various types of Ub modification to the bacterial coat transform bacterial surfaces into signalling platforms. For example, linear Ub chains not only attract the autophagic machinery but also locally activate NF-kB signalling for a maximal antibacterial response[165,166]. Notably, many pathogens have evolved strategies to escape the autophagic machinery by secreting factors that interfere with autophagosome maturation[167], blocking fusion of the autophagosome with the lysosome[168] and competing with host autophagy receptors for binding to LC3 (REF.[169]) and so on. Some bacteria even manipulate autophagy for their own benefit and are able to replicate effectively within autophagosome-like vesicles[170]. Nevertheless, autophagy activation is considered a valid therapeutic strategy to combat bacterial infections (BOX 2; TABLE 2).

Conclusions and perspectives

Autophagy currently enjoys star status in cell biology. Initially described as a nonselective mechanism for intracellular garbage disposal and recycling, autophagy has emerged as a highly selective and powerful programme that is critically implicated in various fundamental cellular processes. The cytoprotective properties of autophagy have raised the particular interest of scientists and clinicians. However, initial excitement about therapeutic targeting of autophagy in cancer and other diseases has given way to a sober, more realistic view of autophagy as a druggable process (BOX 2). The uncovering of diverse and sometimes unexpected challenges demands an unbiased re-evaluation of therapeutic strategies. The major task seems to be mapping the context-dependent functional networks in which autophagy is embedded. The challenge is therefore to modulate autophagy without adversely affecting other cellular processes. The fact that autophagy crosstalks with virtually every other cellular system may give some idea of the vast scope of this task, yet it also indicates the enormous potential for beneficial modulation that we can expect to find while exploring this fundamental pathway.

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Review

Mitophagy in Alzheimer's Disease and Other Age-Related Neurodegenerative Diseases

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Abstract: Mitochondrial dysfunction is a central aspect of aging and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease. Mitochondria are the main cellular energy powerhouses, supplying most of ATP by oxidative phosphorylation, which is required to fuel essential neuronal functions. Efficient removal of aged and dysfunctional mitochondria through mitophagy, a cargo-selective autophagy, is crucial for mitochondrial maintenance and neuronal health. Mechanistic studies into mitophagy have highlighted an integrated and elaborate cellular network that can regulate mitochondrial turnover. In this review, we provide an updated overview of the recent discoveries and advancements on the mitophagy pathways and discuss the molecular mechanisms underlying mitophagy defects in Alzheimer's disease and other age-related neurodegenerative diseases, as well as the therapeutic potential of mitophagy-enhancing strategies to combat these disorders.

Keywords: mitophagy; mitophagosome; lysosome; mitochondrial dynamics; mitochondrial quality control; Alzheimer's disease; Parkinson's disease; Huntington's disease; amyotrophic lateral sclerosis; aging

1. Introduction

Mitochondria are termed the "powerhouses" of the cell, and generate the majority of the cell's supply of adenosine triphosphate (ATP) through the oxidative phosphorylation system (OXPHOS) in which electrons produced by the citric acid cycle are transferred down the mitochondrial respiratory complexes. Neurons have particularly high and continuous energy demands so that mitochondrial function is essential for maintaining neuronal integrity and responsiveness [1–7]. Mitochondrial energy production fuels various critical neuronal functions, especially the ATP-dependent neurotransmission [1,3,8]. Along with regulating energy levels, mitochondria have a high capacity to sequester excessive Ca^{2+} and release Ca^{2+} so as to prolong residual levels at synaptic terminals [9,10]. Through this mechanism, mitochondria play essential roles in maintaining and regulating neurotransmission [11,12], as well as certain types of short-term synaptic plasticity [13,14]. In addition, mounting evidence has demonstrated the critical role of mitochondria in the maintenance of cellular homeostasis [15]. Glucose was shown to be an efficient energy source in neurons and glia that can consume energy produced in parallel by glycolysis and OXPHOS. However, upon neural network activation, the energy demand is robustly enhanced. Given ATP as the main energy source in neurons, mitochondrial energy metabolism thus may play a major role in supplying ATP to fuel these neuronal activities. Importantly, distinct mitochondrial energetic status might also have a significant impact on the cellular signaling pathways. Aged and dysfunctional mitochondria are defective in ATP production and Ca^{2+} buffering, leading to energy deficit and interruptions of neuronal function and health. Furthermore, damaged mitochondria trigger concomitant leakage of electrons and thus promote the production of harmful reactive oxygen species

(ROS) that can damage nucleic acids, proteins, and membrane lipids [1,16–18]. Moreover, mitochondrial oxidative stress leads to the release of cytochrome *c*, a mitochondrial intermembrane space protein, into the cytosol, inducing DNA damage, caspase activation, and apoptosis [6].

A large body of work suggests that mitochondrial dysfunction underlies cognitive decline in neuronal aging and is one of the most notable hallmarks of age-associated neurodegenerative diseases. Mitochondrial damage causes energy deficit, oxidative stress, and impaired cellular signaling, which has been linked to the pathogenesis of neurodegeneration diseases [2,19–21]. Given that a mitochondrion's half-life is estimated to be about 30 days [22,23], cells have developed the interconnected and elaborate pathways through the balance of mitochondrial biogenesis and efficient removal of damaged mitochondria to ensure the maintenance of mitochondrial integrity and bioenergetic functions. Mitophagy, a selective form of autophagy, constitutes a key pathway of mitochondrial quality control mechanisms involving sequestration of defective mitochondria into autophagosomes for subsequent lysosomal degradation [1,2,24,25]. Disruption of mitophagy has been indicated in aging and various diseases, including neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) [7,26]. This review aims to provide a thorough and timely overview of the mitophagy pathways, summarize the underlying mechanisms of mitophagy defects in AD and other age-related neurodegenerative diseases, and highlight the possible therapeutic strategies targeting mitophagy towards confronting mitochondrial dysfunction and neurodegeneration.

2. Overview of the Mitophagy Pathways

Mitophagy (mitochondrial autophagy) is the only known cellular pathway through which entire mitochondria are completely eliminated within lysosomes. Under physiological conditions, mitophagy plays an essential role in the basal mitochondrial turnover and maintenance. More importantly, mitophagy can also be robustly induced in response to a variety of pathological stimuli [7,25–27]. There are a number of mitophagy pathways that have been identified (Figure 1).

2.1. PINK1-Parkin-Mediated Mitophagy

PTEN-induced putative kinase protein 1 (PINK1)-Parkin-mediated mitophagy is the most heavily studied and the best-understood mitophagy pathway [28–30]. In brief, loss of mitochondrial membrane potential ($\Delta\psi_m$) accumulates PINK1 on the outer membrane of mitochondria (OMM) to recruit and activate Parkin, an E3 ubiquitin ligase, through phosphorylation of ubiquitin [31–38]. Parkin then ubiquitinates a number of OMM proteins and subsequently activates the ubiquitin-proteasome system (UPS) to degrade these ubiquitinated OMM proteins [39–43]. This leads to recruitment of the autophagy machinery to promote the engulfment of damaged mitochondria by phagophore or isolation membranes and thus formation of mitophagosomes destined for removal via the lysosomal system. The roles of PINK1 and Parkin in mitochondrial quality control and mitophagy have been supported by multiple studies in *Drosophila* [28,44–47]. The PINK1-Parkin pathway was shown to facilitate mitophagy as well as selective mitochondrial respiratory chain turnover [28,44–47]. Furthermore, genetic and clinical data have provided clear evidence to support the notion that the PINK1-Parkin pathway is involved in the pathogenesis of PD [48,49]. However, recent in vivo studies indicate that PINK1 and Parkin are not critical for basal mitophagy in a range of tissues including the brain [50,51]. More recent studies have been focused on understanding the PINK1-Parkin-independent mitophagy pathways.

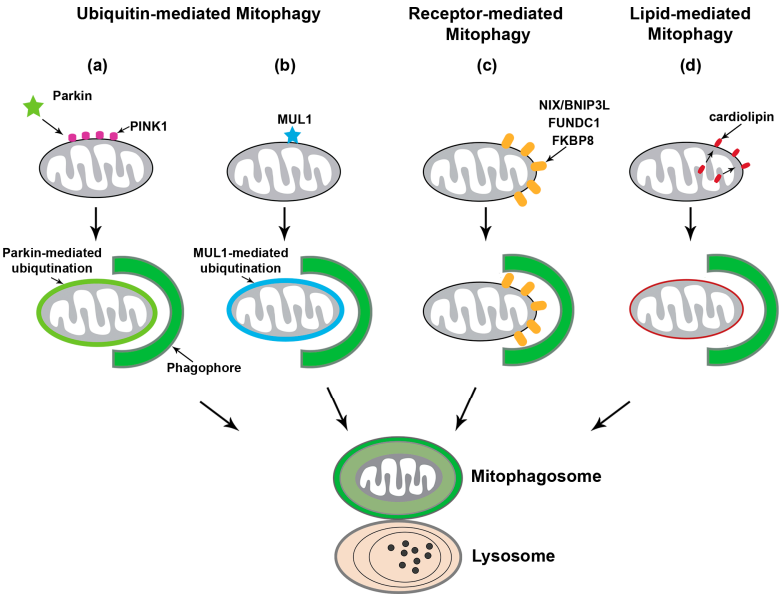

Figure 1. The mitophagy pathways. Upon mitochondrial damage, mitophagy can be induced through three major mechanisms: ubiquitin-mediated mitophagy including PTEN-induced putative kinase protein 1 (PINK1)-Parkin-dependent mitophagy, outer mitochondrial membrane (OMM) receptor-mediated mitophagy, and lipid-mediated mitophagy. (**a**) PINK1-Parkin-mediated mitophagy initiates with PINK1 stabilization on the OMM of damaged mitochondria to recruit Parkin, an E3 ubiquitin ligase. Phospho-ubiquitination of substrates on the OMM by PINK1/Parkin recruits the autophagy machinery and thus promotes the engulfment of damaged mitochondria by growing phagophore or isolation membranes. (**b**) Other E3 ubiquitin ligases have been reported to regulate mitophagy independent of PINK1 and Parkin. MUL1 has similar substrates to Parkin and can directly bind to GABAA receptor-associated protein (GABARAP), suggesting that MUL1 can function independently to facilitate autophagic engulfment. (**c**) Several autophagy receptors are anchored within the OMM, including NIP3-like protein X (NIX; also known as BNIP3L), FUN14 domain containing 1 (FUNDC1), and FK506 Binding Protein 8 (FKBP8). Binding of NIX/BNIP3L or FUNDC1 to LC3 or GABARAP on the phagophore mediates targeting dysfunctional mitochondria for autophagy. (**d**) Externalization of cardiolipin, normally found in the inner mitochondrial membrane (IMM) phospholipid, to the OMM is a unique mechanism for lipid-mediated mitophagy. Cardiolipin initiates mitophagy through its direct interaction with LC3.

2.2. *Ubiquitin-Mediated Mitophagy Independent of Parkin*

Other E3 ubiquitin ligases that can also mediate removal dysfunctional mitochondria have been identified [52], which is with relation to PINK1-Parkin-independent mitophagy mechanisms. Mitochondrial ubiquitin ligase 1 (MUL1, also known as MAPL, GIDE, and MULAN) was reported to play a role in the regulation of mitophagy through multiple mechanisms. MUL1 interacts with mitochondrial fission GTPase protein dynamin-related protein 1 (Drp1) and mitochondrial fusion protein Mitofusin, both of which are the substrates of Parkin [53,54]. MUL1 has no effect on PINK1-Parkin-mediated mitophagy, but can suppress PINK1 or Parkin mutant phenotypes in both *Drosophila* and mouse neurons. This suppression is attributed to the ubiquitin-dependent degradation of Mitofusin. Interestingly, double mutants of MUL1 with either PINK1 or Parkin show much more severe phenotypes. Moreover, MUL1 contains an LC3-interacting region (LIR) motif in the RING domain through which MUL1 interacts with GABAA receptor-associated protein (GABARAP), a member of the Atg8 family that plays a key role in autophagy and mitophagy [55]. Thus, these observations collectively suggest that MUL1 functions in a pathway parallel to the PINK1-Parkin pathway. In addition to MUL1, a recent study reported PINK1-synphilin-1-SIAH-1 as another newly discovered Parkin-independent pathway that can promote PINK1-dependent mitophagy in the absence of Parkin [56].

2.3. Receptor-Mediated Mitophagy

The BCL-2 homology 3 (BH3)-containing protein NIP3-like X (NIX, also known as BNIP3L), an OMM protein, was reported to play an important role in mitochondrial turnover in erythrocytes [57]. NIX/BNIP3L contains an LIR motif at the amino-terminal that binds to LC3 on phagophore or isolation membranes, and is transcriptionally upregulated during erythrocyte differentiation [58]. Such a mechanism enables NIX/BNIP3L to serve as a selective mitophagy receptor and promote recruitment of the autophagy machinery to the surface of damaged mitochondria in erythroid cells. NIX/BNIP3L was also reported to be involved in hypoxia-induced mitophagy, during which forkhead box O3 (FOXO3) and hypoxia-inducible factor (HIF) transcriptionally regulate NIX/BNIP3L along with BNIP3 [59]. Noteworthy, overexpression of NIX/BNIP3L can restore mitophagy in skin fibroblasts from PD patients carrying mutations in *PARK6* or *PARK2* [60], suggesting an independent role of NIX/BNIP3L in PINK1-Parkin-mediated mitophagy. NIX/BNIP3L and BNIP3 were reported to be upregulated upon neuronal stress [61,62]. However, the extent to which NIX/BNIP3L and BNIP3 might participate in neuronal mitophagy remains unclear. FUN14 domain containing 1 (FUNDC1) also functions as a mitophagy receptor and regulates the autophagic clearance of mitochondria under hypoxic stress. Studies have demonstrated that the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) dephosphorylates FUNDC1 to activate mitophagy during hypoxia [63–65]. Additionally, FK506 Binding Protein 8 (FKBP8) was recently reported to have LIR domains and can mediate Parkin-independent mitophagy by recruiting LC3A [66]. Collectively, these observations suggest that specific mitophagy receptors on the OMM play an essential role in recruiting the autophagy machinery to damaged mitochondria for lysosomal clearance.

2.4. Lipid-Mediated Mitophagy

Recent studies have demonstrated that lipids can also act as an elimination signal to mediate recruitment of injured mitochondria to the autophagy pathway. Apart from ubiquitin- or receptor-mediated mitophagy, this pathway involves the direct interaction of LC3 with the phospholipid cardiolipin, and was originally observed in neuroblastoma cells and primary cortical neurons incubated with rotenone, staurosporine, or 6-hydroxydopamine [67]. Cardiolipin is primarily found in the inner membrane of mitochondria (IMM) and is externalized to the OMM upon mitochondrial damage. Three enzymatic translocations are needed for the externalization of cardiolipin, which are mediated by the phospholipid scramblase-3 of mitochondria and the inner and outer membrane spanning hexameric complex of mitochondrial nucleoside diphosphate kinase D (NDPK-D/NM23-H4) in SH-SY5Y cells or Tafazzin (TAZ) in mouse embryonic fibroblasts (MEFs), respectively [67–69]. Furthermore, cardiolipin interacts with LC3, and this interaction is facilitated by the negatively charged basic residues in LC3 and charged head group of cardiolipin. Thus, cardiolipin-mediated mitophagy is independent of PINK1 and Parkin. Importantly, cardiolipin downregulation or mutagenesis of LC3 at the sites predicted to interact with cardiolipin was shown to impair mitophagosome formation [67]. In addition, genome-wide screens indicate that F-box and WD40 domain protein 7 (FBXW7), sterol regulatory element binding transcription factor 1 (SREBF1), and other components of the lipogenesis pathway may play a role in the regulation of Parkin-mediated mitophagy [70]. Additionally, upon Drp1-mediated mitochondrial fission, ceramide was shown to promote autophagic recruitment of mitochondria through direct interaction of ceramide with LC3B-II [71].

2.5. Neuronal Mitophagy

Neurons are highly polarized cells with unique properties in structure and function. Mitochondrial quality control mechanisms that efficiently sense and eliminate mitochondria damaged over usage, aging, or disease could be critical for neuronal health. Mitophagy is currently believed to constitute the major cellular pathway for mitochondrial quality control in neurons. While basal mitophagy is known to be required for the maintenance of neuronal homeostasis, mounting evidence has shown that mitophagy

can be upregulated in response to various pathological stimuli (Figure 2). Cardiolipin-mediated mitophagy can be induced in primary cortical neurons treated with the mitochondrial complex I inhibitor rotenone [67]. As for Parkin-mediated mitophagy, $\Delta\psi_m$ dissipation triggers Parkin translocation onto depolarized mitochondria in neurons after treatment with CCCP, an $\Delta\psi_m$ uncoupler [72,73]. Interestingly, Parkin-targeted mitochondria primarily accumulate in the somatodendritic region of neurons where they undergo autophagic sequestration for lysosomal degradation. Moreover, mitophagy activation reduces anterograde transport, but increases retrograde transport of axonal mitochondria, suggesting that damaged mitochondria are trafficked back to the soma for mitophagic clearance. Parkin-dependent mitophagy was also discovered under AD-linked pathophysiological conditions in the absence of any $\Delta\psi_m$ dissipating reagent [74]. The spatial aspects of Parkin-dependent mitophagy were also observed in vivo. In particular, the PINK1 and Parkin mutant *Drosophila* exhibit abnormal tubular and reticular mitochondria restricted to the cell body, as well as normal morphology with reduced mitochondrial flux within axons [46,47]. In addition to *Drosophila*, the evidence from examination of Purkinje neurons in the mito-QC reporter mice suggests that the majority of mitochondrial turnover occurs in the Purkinje somata. This supports the view that damaged mitochondria or mitophagosomes are returned to the cell body for lysosomal clearance [75]. Collectively, these in vitro and in vivo observations consistently suggest that the soma is in the focus of neuronal mitophagy, a selective process with a function to restrict damaged mitochondria to the soma and thus limit the impact of impaired mitochondrial function on distal axons.

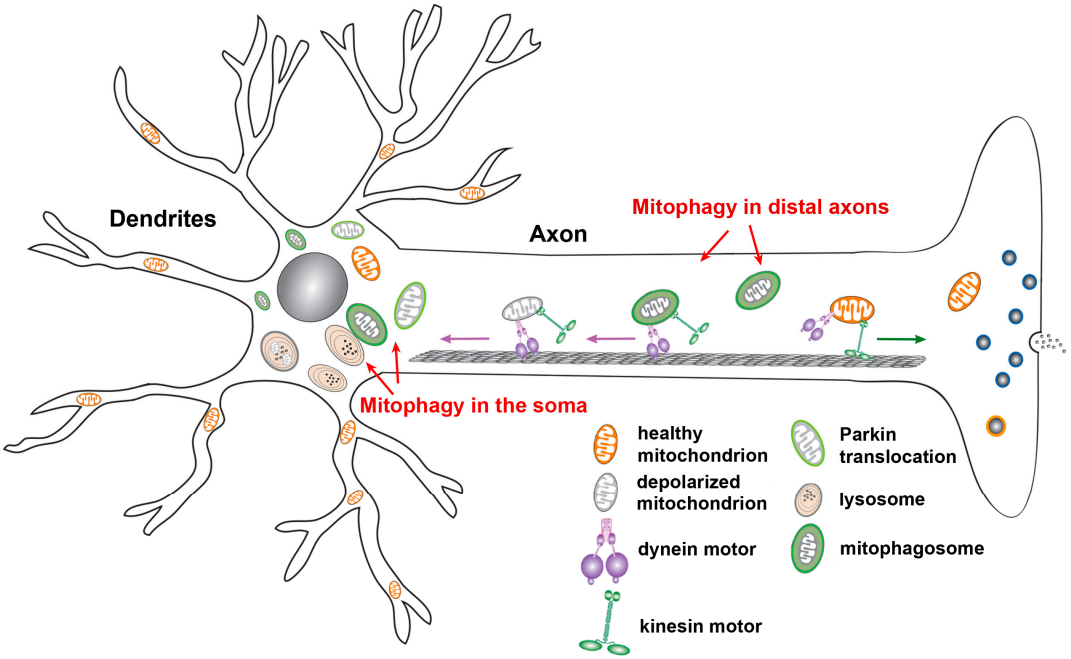

Figure 2. Neuronal mitophagy. Upon mitochondrial membrane potential ($\Delta\psi_m$) dissipation, Parkin is recruited to depolarized mitochondria, triggering mitochondrial engulfment by autophagosomes. Parkin-targeted mitochondria accumulate in the somatodendritic regions of neurons. Such compartmental restriction is attributed to altered mitochondrial motility along axons, as evidenced by decreased anterograde transport and relatively increased retrograde transport of mitochondria. This spatial process allows neurons to efficiently remove damaged mitochondria from axonal terminals and facilitate mitophagic clearance in the soma, where mature lysosomes are mainly located. Studies have also shown that autophagosomes containing engulfed mitochondria move in an exclusively retrograde direction from distal axons toward the soma for maturation and for more efficient cargo degradation within acidic lysosomes in the soma. Figure is modified from [2].

2.6. Mitophagy In Vivo

The mitophagy pathways have been extensively studied *in vitro*. To address the basal mitophagy in vivo, a number of transgenic mice expressing sensors to monitor the delivery of mitochondria to acidic organelles (lysosomes) have been developed [75,76]. These studies have demonstrated active mitochondrial delivery to acidic organelles in multiple tissues but with variable rates. A recent work further shows that the basal mitophagy is independent of the PINK1 pathway [51]. Consistently, studies from *Drosophila* expressing fluorescent mitophagy reporters, either mito-Keima or mito-QC, also reveal robust basal mitophagy in different tissues [50]. However, null mutations of either PINK1 or Parkin do not lead to altered rates of mitochondrial delivery into lysosomes, suggesting nonessential roles of PINK1 and Parkin in the basal mitophagy in vivo. These data are also consistent with the observations from mice with the deletion of *PARK6* or *PARK2*. These mice lack strong phenotypes, such as dopaminergic neuron loss [77–81]. Importantly, the evidence of mitophagy activation is clear in the brain tissues of human patients with neurodegenerative diseases [74,82,83]. Given multiple distinct mechanisms that have been identified to target damaged mitochondria for autophagy, other PINK1-Parkin-independent pathways or other as yet undefined mechanisms likely play more important role in the basal neuronal mitophagy. Therefore, the involvement of these mitophagy pathways in the basal mitochondrial turnover and in response to specific disease-related stressors needs to be carefully determined in vivo.

3. Mitochondrial Dysfunction in Neurodegenerative Diseases

Mitochondrial defects are a significant concern in the aging nervous system and have been consistently linked to age-related neurodegenerative diseases, suggesting that the underlying mechanisms might be somewhat shared (Figure 3).

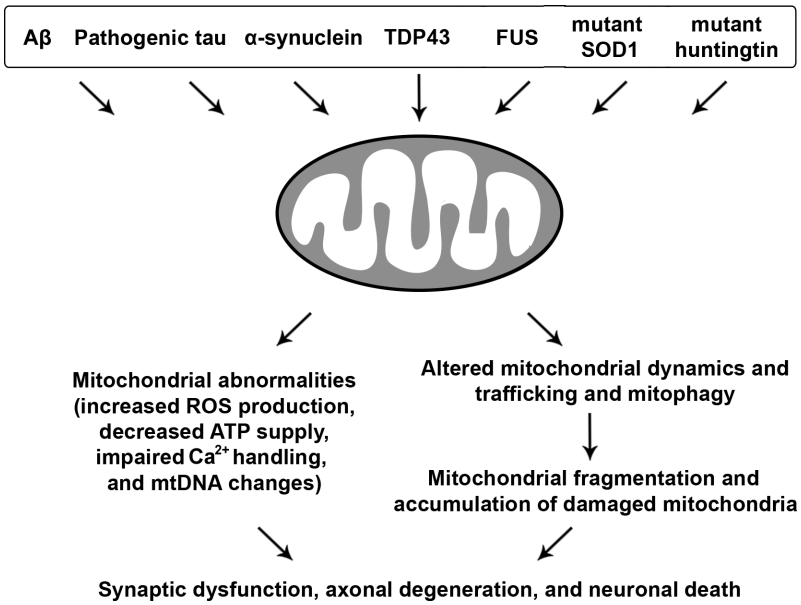

Figure 3. Neurodegenerative disease-associated mitochondrial toxicity. Misfolded proteins, oligomers, protein aggregates, or fibrils linked to major neurodegenerative diseases induce mitochondrial abnormalities, leading to increased reactive oxygen species (ROS) levels, loss of mitochondrial membrane potential ($\Delta\psi_m$), decreased oxidative phosphorylation (OXPHOS) and ATP production, impaired Ca^{2+} buffering, and enhanced mitochondrial DNA (mtDNA) changes. Moreover, impairments in mitochondrial dynamics and trafficking as well as mitophagy result in excessive mitochondrial fission and fragmentation and aberrant accumulation of dysfunctional mitochondria, all of which collectively contribute to synaptic dysfunction, axonal degeneration, and neuronal death.

3.1. Aβ and Tau-Linked Mitochondrial Abnormalities

AD is the most common form of neurodegenerative diseases in aging populations. Progression of the disease involves cognitive decline, memory loss, and neuronal death in the cerebral cortex and subcortical regions. AD patient brains are characterized by extracellular amyloid plaque deposits, composed of agglomerated amyloid β (Aβ) peptides, as well as intracellular accumulation of neurofibrillary tangles (NFTs), consisting of hyperphosphorylated tau (phospho-tau) protein. Mitochondrial disturbances have been suggested as a hallmark of AD as the patients exhibit early metabolic alterations prior to any histopathological or clinical manifestations [84]. Mitochondrial dysfunction, oxidative stress, and mitochondrial DNA (mtDNA) changes are prominent pathological features reported in AD postmortem brains [85–95]. Importantly, a growing body of evidence has indicated a major role of mitochondrial defects in the pathogenesis of AD [2,96–98].

The degree of cognitive dysfunction in AD was linked to the extent of Aβ accumulation within mitochondria and mitochondrial abnormalities [99]. Aβ has been proposed to be a key player in mediating mitochondrial damage. Aβ was found to impair multiple aspects of mitochondrial function [100–102], including function of the electron transport chain (ETC) [103], ROS production [104–106], mitochondrial dynamics [91,103,107,108], and mitochondrial transport [109–111]. The possible routes for Aβ to enter into mitochondria were thought to be through the translocase of the outer membrane (TOM) complex or mitochondrial-associated endoplasmic reticulum (ER) membrane (MAM) [112–115]. In addition to intracellular Aβ, mitochondria can also take up internalized extracellular Aβ [114,116]. Aβ1–42 treatment was shown to lead to the opening of mitochondrial permeability transition pore (mPTP) in cultured cortical neural progenitor cells. While transient mPTP opening decreases cell proliferation, prolonged mPTP opening irreversibly causes cell death [117]. Consistent with this observation, an interesting work in a live AD mouse model provided direct evidence that fragmented and defective mitochondria are limited to the vicinity of extracellular amyloid plaques that likely serve as a focal source to promote abnormal accumulation of Aβ within mitochondria and thus exacerbate Aβ-linked damage [118].

The mechanisms underlying Aβ-mediated mitochondrial toxicity have been carefully investigated by several studies. The interactions of Aβ with Aβ-binding alcohol dehydrogenase (ABAD), a mitochondrial matrix protein, and cyclophilin D (CypD), a component of the mitochondrial transition pore, were reported to mediate Aβ-induced cytotoxic effects [101,102]. In particular, ABAD was shown to be upregulated in AD neurons. Overexpression of ABAD can exacerbate Aβ-induced cellular oxidative stress and cell death. Aβ also forms a complex with CypD in the cortical regions of postmortem human AD patient brains and an AD mouse model [102]. Deletion of CypD in AD mice rescues the mitochondrial phenotypes including impaired Ca^{2+} uptake, mitochondrial swelling due to increased Ca^{2+}, depolarized $\Delta\psi_m$, elevated oxidative stress, decrease in ADP-induced respiration control rate, and reduced complex IV activity and ATP levels. Moreover, CypD deficiency can improve synaptic function as well as learning and memory in an AD mouse model [102]. These observations collectively suggest that Aβ-CypD interaction mediates AD-associated mitochondrial defects. Taken together, these pieces of evidence indicate that the aberrant accumulation of Aβ within mitochondria likely plays a causative role in impaired mitochondrial function in AD.

Pathogenic forms of tau can also induce mitochondrial damage. A number of studies have demonstrated that phospho-tau specifically impairs complex I of the mitochondrial respiratory chain, resulting in increased ROS production, loss of $\Delta\psi_m$, lipid peroxidation, and reduced activities of detoxifying enzymes such as superoxide dismutase (SOD) [119,120]. Overexpression of the mutant human tau protein htauP301L was reported to reduce ATP levels and increase susceptibility to oxidative stress in cultured neuroblastoma cells [121]. Disrupted activity and altered composition of mitochondrial enzymes can also be detected in the P301S mouse model of tauopathy [122]. In the pR5 mice overexpressing the htauP301L, mitochondrial dysfunction was evidenced by impaired mitochondrial respiration and ATP synthesis, decreased complex I activity, and increased ROS levels [123,124]. Phospho-tau was also reported to directly interact with VDAC in AD brains. This

interaction was proposed to impair mitochondrial function likely through blocking mitochondrial pores [125]. Furthermore, mitochondrial stress was shown, in turn, to enhance hyperphosphorylation of tau in a mouse model lacking SOD2 [126]. Inhibition of mitochondrial complex I activity reduces ATP levels, resulting in a redistribution of tau from the axon to the soma and subsequent cell death [127]. Thus, these observations suggest that the toxic effects of tau on mitochondria could be reciprocal and that mitochondrial deficiency might play a critical role in the development of tau pathology.

Both Aβ and pathogenic tau have deleterious effects on mitochondrial dynamics through which impact mitochondrial function. Studies on postmortem brain tissues from human patients with AD and mouse models have demonstrated increased levels of Drp1 and Fis1 and reduced levels of mitofusin 1 (Mfn1), Mfn2, and OPA1. Moreover, Aβ overproduction, phospho-tau accumulation, as well as abnormal interactions of Drp1 with Aβ or phospho-tau cause excessive mitochondrial fission and fragmentation, which tend to increase as AD progresses [91,97,108,125,128]. Cells overexpressing mutant tau associated with frontotemporal dementia (FTD) with Parkinsonism linked to chromosome 17 (FTDP-17) display decreased rates of mitochondrial fusion and fission and enhanced vulnerability to oxidative stress [121]. Strikingly, reduction of Drp1 expression can protect against mutated tau-induced mitochondrial dysfunction [129]. Collectively, these data suggest that the pathogenic forms of tau and Aβ could impair mitochondrial function either through direct interaction with VDAC, ABAD, or CypD, or indirectly through their toxic effects on mitochondrial dynamics.

3.2. Mitochondrial Defects with Synucleinopathies

PD is the second most common form of neurodegenerative disease, which is characterized by the aberrant accumulation of α-synuclein (α-syn) in the form of Lewy bodies, especially in the substantia nigra. α-syn is abundant throughout the central nervous system and Lewy bodies are a defining feature of many clinical phenotypes known as synucleinopathies [130,131]. Importantly, impaired mitochondrial function is also a pathological feature of both sporadic and familial PD [20,28,44,49,132–134]. The relationship between α-syn and mitochondria has been explored in many studies. Some evidence showed that mitochondria could be the main targets of α-syn. In particular, the oligomerization and aggregation of α-syn can cause deficits in the complex I activities, leading to reduced ATP levels, depolarized $\Delta\psi_m$, and the release of cytochrome *c* into the cytosol to trigger apoptosis [135,136]. A number of studies have shown that α-syn is directly localized in mitochondria, and can be detected in isolated mitochondria from PD patient brains. Mitochondrial localization of α-syn has a negative impact on mitochondrial function, morphology, and dynamics [137–143]. α-syn has a cryptic mitochondrial targeting sequence located at its amino terminal region through which α-syn is constitutively imported into mitochondria and associates with the IMM. Such a mechanism leads to reduced complex I activities and elevated ROS levels in human dopaminergic neurons [140]. Moreover, oligomeric and dopamine-modified α-syn disrupts the association of the OMM translocase TOM20 and its coreceptor, TOM22, resulting in protein import impairment [144]. Thus, diminished import of mitochondrial proteins impairs mitochondrial function in nigrostriatal neurons, as reflected by deficient respiration, loss of $\Delta\psi_m$, and enhanced production of ROS.

α-syn can also affect mitochondrial dynamics and mitophagy. Basically, α-syn is known to bind to the lipid membranes, especially the lipids of the ER membrane or the MAM through which ER interacts with mitochondria. Mutated α-syn decreases the ER-mitochondria contact or interaction, leading to MAM dysfunction and thus mitochondrial fragmentation [141]. Other studies into PD have demonstrated that α-syn causes mitochondrial fragmentation through either direct binding or as a result of increased Drp1 [142,145]. Cleavage of Opa1 was found in dopaminergic neurons with overexpression of α-syn, resulting in decreased mitochondrial fusion [145]. Consistently, suppression of Drp1-mediated mitochondrial fission was reported to protect cells from α-syn-induced cytotoxicity [146]. In addition, studies have demonstrated the direct binding of α-syn to cardiolipin [147,148]. Furthermore, PD-related SNCA-mutant neurons exhibit increased externalization of cardiolipin to the OMM.

Externalized cardiolipin was shown to bind to and promote refolding α-syn fibrils. Importantly, the exposed cardiolipin initiates LC3 recruitment to mitochondria and thus enhances mitophagic turnover, leading to reduced mitochondrial volume and exacerbation of mutant α-syn-induced mitochondrial dysfunction [148]. On the other hand, mitophagy defects were also proposed to play a significant role in PD pathogenesis, especially augmenting α-syn accumulation and its mediated neurotoxicity [149–151].

3.3. ALS and FTD-Associated Mitochondrial Toxicity

ALS is a devastating disease characterized by motor neuron degeneration. A hallmark of ALS, as in the pathologies of other neurodegenerative diseases, is the abnormal accumulation of misfolded proteins and protein aggregates within the affected motor neurons. FTD affects the basal ganglia and cortical neurons, leading to cognitive deficits, language deficiency along with altered social behavior and conduct. Even though the affected neuron types are quite different, ALS and FTD show the similarities in genetic background and pathological processes and also share the common pathways of neurodegeneration [152,153]. Defects in oxidative phosphorylation, Ca^{2+} buffering, and increased ROS production have been linked to ALS pathogenesis [154]. Multiple studies in cell culture and in transgenic animal models of ALS reveal alterations in oxidative metabolism linked to changes in ETC activity and reduced ATP synthesis [155–158]. More importantly, mitochondria purified from ALS patients display impaired Ca^{2+} homeostasis and increased ROS levels. Such defects are coupled with oxidative damage including altered tyrosine nitration and protein carbonylation [159,160]. Indeed, glutamate-receptor mediated excitotoxicity was linked to overloaded mitochondrial Ca^{2+} and increased ROS levels in spinal motor neurons cultured from an ALS animal model [161].

Aggregation of the transactive response DNA-binding protein 43 kDa (TDP-43) and fused in sarcoma (FUS) is the pathological hallmarks of both ALS and FTD. Both TDP-43 and FUS are ribonuclear proteins and contain the glycine molecule-enriched prion-like domains that can increase the propensity of TDP-43 and FUS for aggregation as well as cell-to-cell transmission. Aged animals expressing mutant FUS exhibit abnormal accumulation of ubiquitin-positive aggregates, which correlates with neuron loss. These aggregates also stain positive for mitochondrial protein cytochrome *c*, suggesting that damaged mitochondria recruit the autophagy machinery for removal through mitophagy [162]. One study from a single postmortem analysis of an FUS mutation carrier uncovered similar defects. Additionally, C- and N-terminal fragments of TDP-43 were identified within mitochondria. Furthermore, animal models of TDP-43 pathology exhibit membranous organelle redistribution and clustering within cytoplasmic inclusions accompanied by morphological and ultrastructural alterations, as well as abnormal mitochondrial dynamics, trafficking, and quality control [163–165]. Thus, these data consistently indicate the phenotypes of mislocalized, fragmented, and defective mitochondria associated with ALS and FTD.

3.4. Mutant Htt-Induced Mitochondrial Damage

HD is a neurodegenerative genetic disease that affects muscle coordination and leads to cognitive decline and psychiatric symptoms [166]. This autosomal dominant inherited neurodegenerative disease is the most common genetic cause of abnormal involuntary movements called chorea, and is characterized by mutations in the huntingtin gene (*HTT*) that result in abnormal expansion of the cytosine–adenine–guanine (CAG) trinucleotide repeats in the *HTT* gene, encoding a polyglutamine (polyQ) tract at the N-terminal region of the huntingtin (Htt) protein. The N-terminus of Htt can be cleaved through protease activity, leading to formation of short and toxic polyQ peptides. The N-terminal fragments of Htt containing the polyQ tracts are more prone to aggregation and accumulate within the inclusions in the nucleus especially in the medium spiny neurons of the striatum [167–171]. Htt was reported to directly bind to Tim 23 on mitochondria, thus preventing the protein import into mitochondria. This defect could be reversed through overexpressing Tim 23 [172,173]. In addition, the aggregate accumulation can disrupt the ETC function [174]. Moreover,

studies in HD patient brains found decreased activities of complex II, complex III, and complex IV. Reduced complex II activity was observed particularly in the striatum of HD patients. Such defects along with reduced ATP production were also demonstrated in other studies, which collectively point towards impaired OXPHOS and disrupted mitochondrial energy metabolism [175]. Importantly, overexpression of complex II reduces the mutant Htt-mediated toxic effects in striatal neurons. Moreover, alterations in mitochondrial dynamics were also reported in the striatum of HD patients, as well as in animal and cell models [176,177]. Such a defect is caused by abnormal interaction of mutant Htt with Drp1, leading to Drp1-enhanced mitochondrial fission and thus mitochondrial fragmentation as well as cellular dysregulation and death [178].

4. Mitophagy Defects in Neurodegenerative Diseases

Neurons have very high demand for ATP. Given that mitochondria are the major producer of ATP within cells, the nervous system is especially sensitive to mitochondrial damage. Inefficient elimination of injured mitochondria through mitophagy could be detrimental to neuronal health. Mitophagy deficit has been indicated in aging and the pathogenesis of age-associated neurodegenerative disorders. Studies into mitophagy suggest that defective mitophagy contributes to impaired mitochondrial function and neurodegeneration (Figure 4).

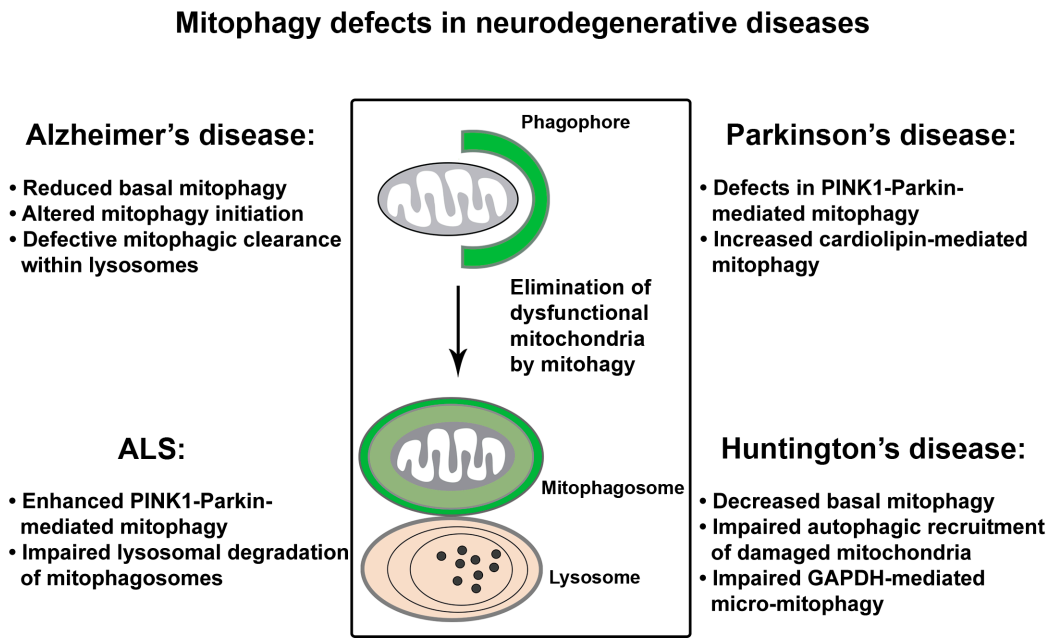

Figure 4. Mitophagy defects in neurodegenerative diseases. A growing body of evidence indicate that mitophagy function is impaired in major neurodegenerative diseases. In Alzheimer's disease (AD), robust activation of Parkin-mediated mitophagy was observed in patient brains of familiar and sporadic AD and the cell and animal models. However, under tauopathy conditions, excessive or defective Parkin-dependent mitochondrial turnover was reported in different studies, respectively. In addition, impaired lysosomal proteolysis and reduced levels of the basal mitophagy collectively contribute to mitophagy dysfunction in AD. In Parkinson's disease (PD), PINK1-Parkin-dependent mitophagy is necessary for the function and survival of PD-related dopaminergic neurons in response to stress/pathological stimuli. The role for increased cardiolipin-mediated mitophagy in mutant α-syn-induced mitochondrial dysfunction has also been proposed. As for amyotrophic lateral sclerosis (ALS), enhanced Parkin-mediated mitophagy was demonstrated in an ALS mouse model. Mitophagy defects and mitochondrial pathology are also attributed to lysosomal deficit in ALS affected motor neurons. In Huntington's disease (HD), decreased basal levels of mitophagy, defects in autophagic recognition and recruitment of damaged mitochondria, and impaired GAPDH-mediated micro-mitophagy lead to pathological mitophagy and mitochondrial dysfunction.

4.1. Mitophagy Defects in AD

Earlier studies revealed abnormal mitophagy in AD patient brains, as evidenced by autophagic accumulation of mitochondria in the soma of vulnerable AD neurons [87,179,180]. Among the multiple distinct mitophagy pathways, PINK1-Parkin-dependent mitophagy has been the focus of current studies in AD. We have shown that the Parkin pathway is robustly induced upon progressive Aβ accumulation and mitochondrial damage in human patient brains and animal models of AD [74]. Furthermore, cytosolic Parkin is depleted in AD brains over the disease's progression, resulting in mitophagic pathology and augmented mitochondrial defects. Consistently, in the AD patient-derived skin fibroblasts and brain biopsies, another study reported diminished Parkin along with abnormal PINK1 accumulation [181]. Mitophagy can be restored in these cells by overexpression of Parkin, as reflected by decreased PINK1 and the recovery of $\Delta\psi_m$ coupled with reduced retention of defective mitochondria. Therefore, these findings indicate that impaired mitochondrial function and abnormal retention of dysfunctional mitochondria could be attributed to mitophagy defects in AD neurons. In addition, cardiolipin cluster-organized profile was shown to be lost in synaptic mitochondria purified from AD mouse models [182], occurring at the early disease stages and before nonsynaptic mitochondrial defects. This data suggests the possibility that cardiolipin-mediated mitophagy might be deficient in AD.

The degradation capacity of lysosomes is critical for mitophagic clearance, and defects in lysosomal proteolysis of autophagic cargoes can also impair the mitophagy function. Lysosomal deficit is a prominent feature in AD brains, linked to the pathogenesis of AD. Suppression of lysosomal proteolysis in wild-type (WT) mice was shown to mimic neuropathology of AD and exacerbate autophagic pathology and amyloidogenesis in AD mouse models [183,184]. Presenilin 1 mutations along with ApoE4, a key genetic risk factor of AD, are thought to disrupt lysosomal function [185]. Other factors, including Aβ peptides, phospho-tau, ROS, and oxidized lipids and lipoproteins, could also impair lysosomal proteolysis and result in a toxic accumulation, thus triggering apoptosis and neuronal death in AD. Our recent study proposes that AD-linked lysosomal deficit is also attributed to defects in protease targeting to lysosomes [186]. It is known that newly synthesized protease precursors need to be delivered from the trans-Golgi network (TGN) to the endo-lysosomal system for maturation, a process that relies on the presence of cation-independent mannose 6-phosphate receptor (CI-MPR) at the Golgi. The retromer complex mediates the retrieval of CI-MPR from late endosomes to the TGN and thus facilitates the trafficking of proteases to late endosomes and lysosomes [187]. Our study reveals that retromer dysfunction and defective CI-MPR recycling to the Golgi lead to defects in protease targeting to lysosomes. As a result, protease deficit within lysosomes impedes lysosomal proteolysis of defective mitochondria along with other autophagic cargoes in AD neurons [186]. Therefore, increased Parkin association with mitochondria, autophagic accumulation, as well as abnormal mitochondrial retention within lysosomes observed in AD neurons of patient brains and in cultured cells overexpressing mutant APP could also represent lysosomal deficiency [74,186]. Taken together, these observations indicate that defective mitophagy is likely involved in AD-linked neurodegeneration.

Pathogenic truncation of tau could impair mitophagy function. A recent work reported a stable association of an NH2-htau fragment with Parkin and Ubiquitin-C-terminal hydrolase L1 (UCHL-1) in cellular and animal AD models and human AD brains, leading to enhanced mitochondrial recruitment of Parkin and UCHL-1 and thus improper mitochondrial turnover [188]. Mitophagy suppression can restore synaptic mitochondrial density and partially, but significantly, protect against neuronal death induced by this NH2-htau. In contrast, another study proposed that human wild type tau (htau) is inserted into the mitochondrial membrane, thus inducing mitophagy impairment [189]. However, in a more recent study, both htau and htauP301L were shown to impair mitophagy in *Caenorhabditis elegans* (*C. elegans*) and neuroblastoma cells by reducing Parkin translocation onto mitochondria through a different mechanism. Instead of changes in the $\Delta\psi_m$ or the cytoskeleton, impaired Parkin recruitment to mitochondria is proposed to be caused by tau-mediated sequestration of Parkin in the

cytosol [190]. Collectively, these data suggest that mitophagy is impaired under tauopathy conditions by distinct mechanisms.

In addition to defects in mitophagic clearance in response to Aβ and tau-induced mitochondrial damage, a recent study reveals a marked decrease in the basal level of mitophagy in postmortem hippocampal tissues from AD patients, cortical neurons derived from AD-induced pluripotent stem cell (iPSC), as well as AD mouse models [191]. This study further demonstrates defects in the activation of ULK1 and TBK1, the autophagy proteins that mediate autophagy/mitophagy initiation, thus leading to impaired mitophagy function. Furthermore, pharmacological reinstallation of mitophagy mitigates amyloid and tau pathologies, resulting in beneficial effects against memory loss in these AD mice. Therefore, these data support the view that defective mitophagy is likely an early event in AD brains and plays a causative role in the development of AD-linked neuropathology [191]. Further studies using neurons derived from iPSCs of sporadic AD or other similar models could be very critical to addressing whether mitophagy dysfunction serves as a key player in Aβ/tau proteinopathies.

4.2. Mitophagy Defects in PD

Dysfunctional mitophagy is closely linked to PD. Many PD-causing genes show the mitochondrial phenotypes [28,137,150,192–197]. In addition, PD patients have increased rates of mtDNA deletion in the substantia nigra, which further associates defective mitochondrial quality control with PD [198]. The important role for mitophagy in PD was first indicated from an ultrastructural study showing autophagic accumulation of mitochondria in neurons of the patients with PD and Lewy Body Dementia (LBD) [83]. Many other studies have demonstrated mitophagy abnormalities in a variety of experimental models representing genetic forms of toxic-environmental PD [67,199–202]. As previously discussed (see Section 2.1), cell-based and mechanistic studies directly link PINK1 and Parkin to mitophagy. However, while loss-of-function mutations in *PARK6* (encoding PINK1) and *PARK2* (encoding Parkin) genes are linked to familial PD [203], the role of the PINK1-Parkin-dependent pathway in vivo remains elusive. The PINK1 and Parkin pathway has been extensively studied in *Drosophila*. Mutant flies show dopaminergic degeneration, reduced lifespan, and locomotive defects [28,44,204–206]. Muscle cells of mutant flies exhibited swollen mitochondria with disrupted cristae, coupled with muscle degeneration [206–209]. *PARK6* KO rats showed dopaminergic loss and motor defects [210]. Importantly, both *Drosophila* and rat model systems show mitochondrial dysfunction. However, mice with the deletion of *PARK6* or *PARK2* do not exhibit robust substantial PD-relevant phenotypes [77,79–81]. This might be due to compensations by other mechanisms that are sufficient enough to maintain neuronal homeostasis under physiological conditions. Strikingly, when crossing *PARK2* KO mice with Mutator mice characterized by accelerated acquisition of mtDNA mutations, the resulting phenotypes have mitochondrial defects as well as dopaminergic neuronal loss [211]. Thus, this observation suggests that Parkin-mutant mice are susceptible to increased mtDNA damage. Given that both impaired mitochondrial function and mitophagy deficit are the upstream of neurodegeneration, the lack of robust phenotypes in mice suggest that the PINK1-Parkin pathway might be dispensable under physiological conditions, yet still necessary in response to stress/pathological stimuli for the functional maintenance and survival of PD-related dopaminergic neurons. Aside from PINK1-Parkin-dependent mitophagy, increased cardiolipin-mediated mitophagy was proposed to play a role in α-syn-induced mitochondrial dysfunction [148].

4.3. Mitophagy Defects in ALS

Impaired mitophagy was proposed to be involved in the denervation of neuromuscular junctions in an ALS mouse model [212]. Additionally, lysosomal dysfunction has been implicated in ALS. A recent work has provided a strong evidence showing that lysosomal deficits play a critical role in autophagy/mitophagy dysfunction and mitochondrial pathology in a mutant SOD1 transgenic mouse model of ALS [213]. Lysosomal deficits result in abnormal accumulation of autophagic vacuoles (AVs) engulfing damaged mitochondria within motor neuron axons of mutant SOD1 mice. More

importantly, rescuing autophagy-lysosomal deficits was shown to enhance mitochondrial turnover, improve motor neuron survival, and ameliorate disease phenotype in mutant SOD1 mice. Given that autophagy/mitophagy is a lysosome-dependent pathway, defective mitophagy and mitochondrial pathology in ALS are attributed to defects in lysosomal proteolysis.

A more recent study uncovers that Parkin-dependent mitophagy is activated in the mutant SOD1 mouse model of ALS [214]. Mitophagy activation is known to induce Parkin-triggered and the UPS-mediated degradation of mitochondrial dynamics proteins Mfn2 and Mitochondrial Rho GTPase (Miro1) [42,215–218]. Consistently, increased mitophagy in the spinal cord of the mutant SOD1 mice is coupled with depletion of Parkin as well as mitochondrial dynamics proteins Mfn2 and Miro1 that are ubiquitinated by Parkin. Interestingly, genetic ablation of *PARK2* protects against muscle denervation and motor neuron loss and attenuates the depletion of mitochondrial dynamics proteins, which delays disease progression and prolongs life span in mutant SOD1 mice. Thus, the results from this study suggest that Parkin could be a disease modifier of ALS, and chronic activation of Parkin-dependent mitophagy augments mitochondrial dysfunction by depleting mitochondrial dynamics proteins. Consistently, several other studies also reported a significant reduction of Miro1 in spinal cord tissue of ALS patients and animal models [219]. Moreover, it was shown that Miro1 reduction induced by ALS-linked mutant SOD is dependent on Parkin [220]. Miro1 is known as a component of the adaptor-motor complex essential for KIF5 motors to drive anterograde transport of mitochondria along axons [8]. Miro1-knockout mice exhibit upper motor neuron degeneration [221]. Thus, ALS-linked mitochondrial trafficking defect is likely caused by Miro1 deficiency as a result of Parkin-dependent enhancement of Miro1 turnover [220].

Compromised mitophagy may also induce ALS. Many of the genes linked to ALS encode proteins that play a critical role in autophagy/mitophagy, including OPTN and p62, as well as their kinase TBK1 [222–224]. However, it is poorly understood how the mutations in these genes are involved in the ALS pathology. Given the phosphorylation of OPTN and p62 by TBK1 to activate autophagy/mitophagy, aberrant accumulation of misfolded proteins and protein aggregates along with impaired mitochondrial turnover may both contribute to ALS-linked mitochondrial dysfunction and motor neuron death. Illuminating the role of these proteins in vivo will be critical in dissecting the molecular and cellular mechanisms leading to axonal degeneration and motor neuron loss.

4.4. Mitophagy Defects in HD

Mitochondrial dysfunction and autophagy failure have been linked to the pathogenesis of HD. Mutant Htt is known to be associated with mitochondria and to mediate mitochondrial damage. Defective mitophagy may also be involved in mitochondrial defects in HD. Decreased levels of the basal mitophagy were shown in the dentate gyrus of HD mice crossed with the mito-Keima mouse line [76]. In addition to its role in catalyzing the sixth step of glycolysis, a recent study proposed that GAPDH functions in micro-mitophagy—the direct engulfment of injured mitochondria by lysosomes [225]. In HD cell models, abnormal interaction of long polyQ tracts with mitochondrial GAPDH impairs GAPDH-mediated mitophagy, leading to mitochondrial dysfunction and increased cell death. Additionally, mutant Htt can interact with and affect the autophagy machinery [226]. A primary defect in the ability of autophagosomes to recognize and recruit cytosolic cargoes was reported in HD cells, leading to inefficient autophagic engulfment of substrates including mitochondria. Such a defect contributes to HD-associated accumulation of abnormal mitochondria [227]. Moreover, Htt was proposed to act as a scaffold protein for autophagy through the physical interaction of Htt with p62 and ULK1 proteins. This interaction allows Htt to facilitate p62-mediated cargo recognition efficiency, in particular, associating Lys-63-linked ubiquitin-modified substrates with LC3-II—the integral component of phagophore or isolation membranes. Thus, this study supports the possibility that polyQ expansion might compromise the role of Htt in autophagy [228]. Given the evidence for HD-linked autophagy impairment and mitophagic pathology, investigations into mitophagy status as well as detailed mechanisms are important for better understanding of HD pathogenesis.

5. Mitophagy-Targeted Therapeutic Interventions

From the above stated, it is clear that mitochondrial damage is a hallmark of major neurodegenerative diseases. Pharmacological agents that induce mitophagy with a goal of enhancing clearance of damaged mitochondria could be a promising strategy for achieving a significant therapeutic benefit [7,98,229,230]. Several mitophagy inducers, including NAD^+ precursors, urolithin A (UA), the antibiotic actinonin (AC), and spermidine [231,232], have been examined and shown significant benefits in enhancing mitophagy, increasing mitochondrial resistance to oxidative stress, prolonging health span, and for neuronal protection in disease animal models and/or human cells. The levels of NAD^+ are reduced in AD animal models, and elevation of cellular NAD^+ levels through supplementation with NAD^+ precursors such as nicotinamide, nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR) is found to attenuate Aβ and tau pathologies and protect against cognitive dysfunction [233]. Such beneficial effects are attributed to the enhancement of the NAD^+-dependent SIRT1 and SIRT3, expression of the transcription factor CREB, and enhanced activities of PI3K-Akt and MAPK/ERK1/2 [191,233–236]. Additionally, NAD^+ replenishment was also shown to restore mitochondrial function and thus ameliorate dopaminergic neuron loss in iPSC and *Drosophila* models of PD [237]. These observations collectively indicate that the interventions to sustain NAD^+ levels might be beneficial for AD and PD patients.

UA is an ellagitannins-derived metabolite, and can effectively induce neuronal mitophagy in both *C. elegans* and mouse brains [191]. Both UA and AC-mediated mitophagy activation is dependent on PINK1, Parkin, and NIX, and was shown to attenuate AD pathologies, inflammation, and learning and memory deficits [191]. Polyamines, including spermidine, can increase autophagy activity through affecting autophagy-related gene expression as well as enhance mitophagy through the mechanisms of mammalian target of rapamycin (mTOR) inhibition and 5′ AMP-activated protein kinase (AMPK) activation [232,238]. Moreover, spermidine was shown to activate the Ataxia-Telangiesctasia mutated (ATM)-dependent PINK1/Parkin signaling. Treatment with spermidine can lead to memory improvement and prolonged life span observed in *C. elegans*, *Drosophila*, and mice [231,239].

Other pharmacological strategies to enhance mitophagy through inducing mild bioenergetic stress or inhibiting mTOR activity have also been proven to be beneficial in either delaying or treating AD. Mitochondrial uncoupling agents such as 2,4-dinitrophenol (DNP) were reported to stimulate autophagy and preserve neuronal function in AD animal models [240]. Through inducing mild bioenergetic stress and stimulating ketogenesis, 2-deoxyglucose treatment was found to protect neurons against degeneration in a mitochondrial toxin-based PD model [241], as well as enhance mitochondrial function and stimulate autophagic clearance of Aβ [242]. The mTOR inhibitor rapamycin-mediated enhancement of autophagy/mitophagy and AMPK activation can induce mitochondrial clearance in a number of model organisms, including *C. elegans*, *Drosophila*, and mice [243,244]. Rapamycin was also shown to reduce Aβ pathology and ameliorate cognitive dysfunction in a mutant APP transgenic mouse model [245]. Similar to rapamycin, metformin can also stimulate mitophagy through inhibitions of mTOR and complex I activities and activations of AMPK, SIRT1, and Parkin-dependent mitophagy [246–248]. Therefore, these observations collectively provide a strong rationale for future research into the compounds that can enhance mitophagy in AD models, such as UA, AC, and spermidine [76,249,250].

In addition, mitophagy enhancement through activation of Parkin could be another promising strategy in some disease models. Nilotinib was originally identified as a tyrosine kinase inhibitor, and was recently reported to increase Parkin abundance and ubiquitination potentially through enhancing Parkin recycling via the proteasome system [251]. Nilotinib-mediated c-ABL inhibition can also prevent tyrosine phosphorylation of Parkin, leading to the release of Parkin auto-inhibition status. Such a mechanism was demonstrated to be protective in PD models [252]. Moreover, chronic treatment with nilotinib in APP transgenic mice can enhance Aβ clearance through increasing the interaction of Parkin with Beclin 1 [253]. As for ALS and FTD, nilotinib treatment was also reported to mitigate motor and cognitive deficits in TDP-43 transgenic mice [254].

6. Concluding Remarks

Mitochondrial health is vital for cellular and organismal homeostasis, and mitochondrial defects have long been linked to the pathogenesis of neurodegenerative diseases such as AD, PD, ALS, HD, and others. However, it is still unclear whether cellular mechanisms required for the maintenance of mitochondrial integrity and function are deficient in these diseases, thus exacerbating mitochondrial pathology. The quality control of mitochondria involves multiple levels of strategies to protect against mitochondrial damage and maintain a healthy mitochondrial population within cells. In neurons, mitophagy serves as a major pathway of the quality control mechanisms for the removal of aged and defective mitochondria through lysosomal proteolysis. The molecular and cellular mechanisms that govern mitophagy have been extensively studied in the past decade. However, mitophagy deficit has only been recognized recently as a key player involved in aging and neurodegeneration. Given the fact that mitochondrial deficit is clearly linked to neuronal dysfunction and the exacerbation of disease defects, protection of mitochondrial function could be a practical strategy to promote neuroprotection and modify disease pathology. Mitochondrially targeted antioxidants have been proposed. In particular, the antioxidant MitoQ, a redox active ubiquinone targeted to mitochondria, has been examined and demonstrated to have positive effects in multiple models of aging and neurodegenerative disorders [255–258]. Importantly, mitophagy could be another promising target for drug discovery strategy. Therefore, further detailed studies to elucidate mitophagy mechanisms not only advance our understanding of the mitochondrial phenotypes and disease pathogenesis, but also suggest potential therapeutic strategies to combat neurodegenerative diseases.

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Review

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The rapidly evolving view of lysosomal storage diseases

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Abstract

Lysosomal storage diseases are a group of metabolic disorders caused by deficiencies of several components of lysosomal function. Most commonly affected are lysosomal hydrolases, which are involved in the breakdown and recycling of a variety of complex molecules and cellular structures. The understanding of lysosomal biology has progressively improved over time. Lysosomes are no longer viewed as organelles exclusively involved in catabolic pathways, but rather as highly dynamic elements of the autophagic-lysosomal pathway, involved in multiple cellular functions, including signaling, and able to adapt to environmental stimuli. This refined vision of lysosomes has substantially impacted on our understanding of the pathophysiology of lysosomal disorders. It is now clear that substrate accumulation triggers complex pathogenetic cascades that are responsible for disease pathology, such as aberrant vesicle trafficking, impairment of autophagy, dysregulation of signaling pathways, abnormalities of calcium homeostasis, and mitochondrial dysfunction. Novel technologies, in most cases based on high-throughput approaches, have significantly contributed to the characterization of lysosomal biology or lysosomal dysfunction and have the potential to facilitate diagnostic processes, and to enable the identification of new therapeutic targets.

Keywords autophagy; lysosomal biology; lysosomal storage diseases; lysosomes

Subject Categories Autophagy & Cell Death; Genetics, Gene Therapy & Genetic Disease; Organelles

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See the Glossary for abbreviations used in this article.

Introduction

Knowledge on lysosomal storage diseases (LSDs) has been evolving for more than a century (Fig 1). The first phenotypes and clinical entities were described in the 19th century (see Mehta *et al*, 2006 for review), long before the identification of lysosomes and the definition of their biochemistry and pathophysiology. At that time, the identification of these disorders was exclusively based on the characterization of clinical phenotypes and pathology.

The biochemical and cellular bases of LSDs were elucidated much later, when Christian de Duve's work, corroborated by Alex B. Novikoff's electron microscopy observations, led to the identification of lysosomes as cellular catabolic stations (de Duve *et al*, 1955; Novikoff *et al*, 1956), and when the biochemical defects underlying some of the previously described clinical entities were discovered. Pompe disease was the first disorder to be identified as an LSD in 1963, when Henri G. Hers demonstrated that this disease is due to the lack of an acidic α-glucosidase, similar to rat liver lysosomal maltase (Hers, 1963), and that this deficiency is responsible for glycogen storage in tissues. He also suggested that other diseases, such as the mucopolysaccharide storage diseases, might be due to enzyme deficiencies. Between 1960 and the mid-70s, the biochemistry of LSDs was further characterized with the identification of the primary storage materials for other LSDs and the recognition of the respective enzyme deficiencies (Van Hoof, 1974).

For decades, the biology and function of lysosomes remained associated with their catabolic function, and LSD pathophysiology was seen as a direct consequence of defective degradation and disposal of complex substrates (Vellodi, 2005; Heard *et al*, 2010).

Between the late 1970s and 1990s, research in this field progressed with studies that considerably expanded the knowledge on lysosome biology and on the pathophysiology of LSDs. These studies led to the characterization of the mechanism underlying the sorting of lysosomal enzymes (Sly & Fisher, 1982) and to the identification of the molecular bases of clinical variability of LSDs (Beck, 2001; Kroos *et al*, 2012).

Following the recognition of the mannose-6-phosphate pathway's role in lysosomal enzyme trafficking and the availability of new technologies for the purification and manufacturing of lysosomal enzymes, the early 1990s inaugurated the first attempts to treat these disorders by replacing the defective enzyme activity (Barton *et al*, 1990; Barton *et al*, 1991).

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Glossary

Autophagy
A multistep and regulated pathway that removes unnecessary or dysfunctional cellular components and allows for delivery of cargo materials to lysosomes, where they are degraded and recycled.

High-content imaging
Cell-based technologies based on automated microscopy and complex image algorithms to extract multidimensional information on cell morphology, fluorescence intensity, or distribution of fluorescent markers within cells.

Genome editing and CRISPR-Cas9
An RNA-guided targeted genome editing tool: This methodology which allows to introduce different specific genetic changes such as gene knock-out, knock-in, insertions, and deletions in cell lines and in vivo.

Lysosomes
Membrane-limited, ubiquitous, intracellular organelles involved in multiple cellular processes, such as catabolism and recycling of complex molecules and cellular components, signaling, and adaptation to environmental stimuli.

Metabolome analyses
High-throughput methodologies for the detection of multiple metabolites, mainly based on mass spectrometry or nuclear magnetic resonance-based approaches.

Next-generation sequencing
A diagnostic tool based on technological platforms that allow for sequencing of millions of small fragments of DNA in parallel. Next-generation sequencing can be used either for "targeted" sequencing of selected gene panels or for "untargeted" approaches based on whole-exome or genome analysis.

MicroRNAs
Small non-coding RNAs that that regulate gene expression by targeting messenger RNAs.

At the same time, the introduction of novel technologies had a critical role in the study of LSDs. Techniques for targeted gene disruption and generation of knock-out animal models for human LSDs provided tools to better characterize the pathophysiology of these disorders and to develop innovative therapeutic strategies (Pastores *et al*, 2013). Mass spectrometry technology allowed for lysosomal proteome analyses (e.g., mannose-6-phosphate glycoproteome) (Sleat *et al*, 2005) that led to the identification of lysosomal proteins and novel molecular bases of some LSDs.

In the past decade, a number of studies have expanded our knowledge of lysosomal biology and provided new and important insights on LSD pathophysiology. These studies identified the lysosomes as highly dynamic organelles involved in multiple cellular functions, including signaling, and able to adapt to environmental stimuli. (Settembre *et al*, 2013; Perera & Zoncu, 2016; Ballabio & Bonifacino, 2020).

Significant advancements have also facilitated greater understanding on the molecular and metabolic mechanisms underlying LSDs and the development of new therapeutic strategies for these diseases (Parenti *et al*, 2015; Platt *et al*, 2018; Ren & Wang, 2020). This review will focus on new tools and technologies to study LSDs, on emerging aspects of lysosomal biology, and on recent discoveries on the cellular and organismal consequences of lysosomal dysfunction.

The biology of lysosomes, old concepts and new views

Lysosomes are membrane-limited, ubiquitous, intracellular organelles involved in multiple cellular processes (Saftig & Klumperman, 2009; Ballabio & Bonifacino, 2020).

More than two hundred lysosomal-resident proteins contribute to the biology and function of these organelles. Approximately 60 of them are acidic hydrolases (Lubke *et al*, 2009; Schroder *et al*, 2010). Most of them act as exoglycosidases or sulfatases and are localized to the lysosomal lumen. The others are localized at the lysosomal membrane and have multiple functions such as formation of a glycocalyx-like layer, transport across the membrane, acidification, membrane stability, and mediating interaction between lysosomes and other cellular structures (Saftig & Klumperman, 2009; Ballabio & Bonifacino, 2020). In addition to lysosomal-resident proteins, other proteins interact with the lysosome and participate to lysosomal function by being dynamically recruited to the lysosomal surface under certain conditions, for example, the transcription factor EB (TFEB), the mechanistic target of rapamycin complex 1 (mTORC1) (Perera & Zoncu, 2016; Ballabio & Bonifacino, 2020; Yim & Mizushima, 2020), the mTORC1 regulator tuberous sclerosis complex (TSC) (Dibble & Cantley, 2015), folliculin (FLCN) and FLCN-interacting protein (FNIP) (Lawrence *et al*, 2019), the energy-sensing complex AMP-activated kinase (AMPK) (Zhang *et al*, 2014), and the signal transducer and activator of transcription-3 (STAT3) (Liu *et al*, 2018).

The first function of normal lysosomes to be recognized is turnover of cellular constituents. Lysosomes are involved in the degradation of a broad variety of structurally diverse compounds, such as proteins, glycosaminoglycans, sphingolipids, oligosaccharides, glycogen, nucleic acids, and complex lipids. Cellular and extracellular materials and substrates destined for degradation reach lysosomes through different routes (endocytosis, phagocytosis, autophagy), or by direct transport. In this respect, lysosomes are part of a more complex pathway, referred to as the autophagy–lysosomal pathway (ALP). Autophagy plays a crucial role in cell homeostasis by controlling intracellular clearance and recycling of a variety of molecules and cellular components and also by sustaining cellular energy metabolism. Autophagy is a multistep pathway that involves autophagosome formation, cargo recruitment, and autophagosome–lysosome fusion. Importantly, autophagic function is entirely dependent on the ability of the lysosome to degrade and recycle autophagy substrates (Yim & Mizushima, 2020).

Much attention has been paid in recent years to the nutrient-sensing function of lysosomes. Lysosomes are able to monitor the

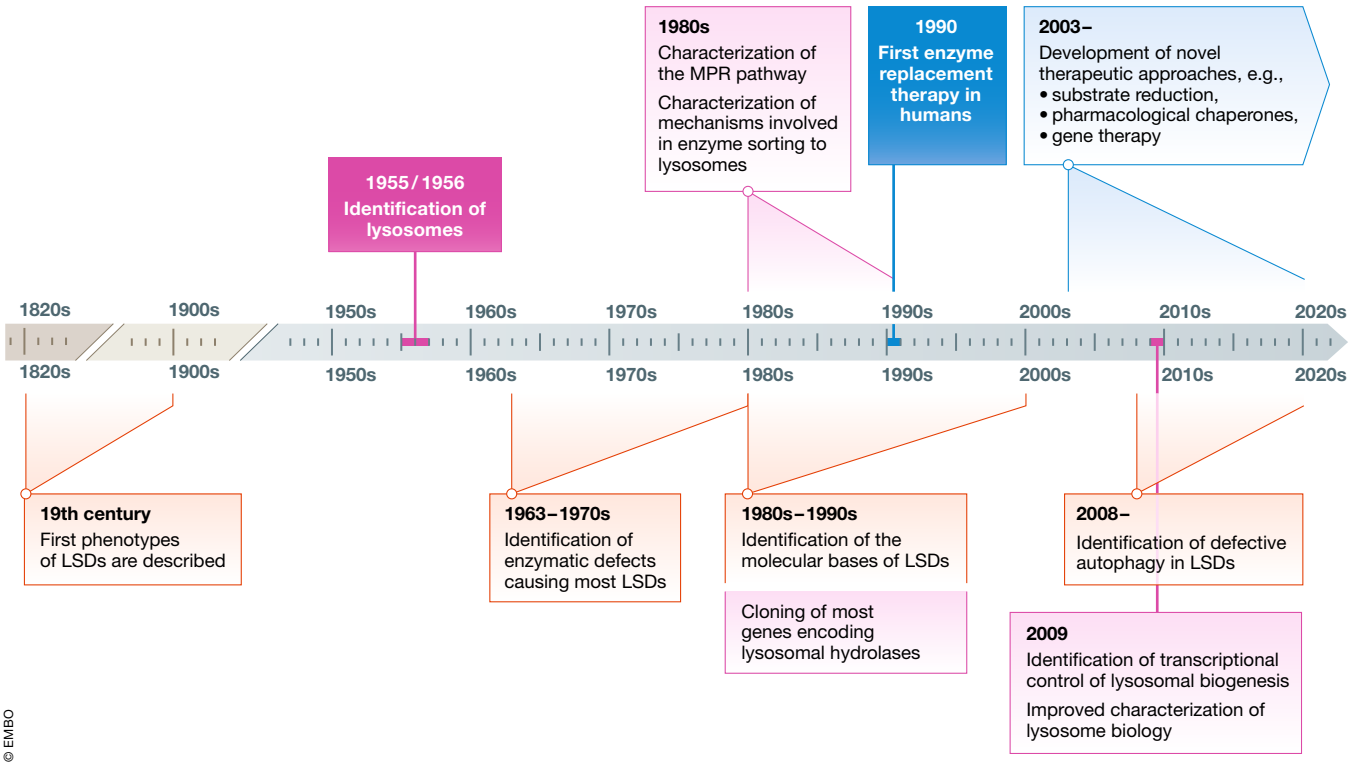

Figure 1. The evolution of the knowledge on LSDs.

After the identification of the first clinical phenotypes during of the 19th century, the knowledge on LSDs evolved following the recognition of lysosomes in 1955/56 and the demonstration of the biochemical defects underlying LSDs, starting from 1963. Between the 1970s and 1990s, research in this field was focused on the mannose-6-phosphate receptor pathway and on the mechanisms underlying the sorting of lysosomal enzymes, on the identification of the molecular bases of LSDs, and on the development of tools and strategies to investigate lysosomal biology. The first attempts to treat these disorders by enzyme replacement therapy started in the 1990s. Current research is now focusing on the role of lysosomes as signaling platforms controlling cellular metabolism and on the development of new therapeutic approaches.

nutrient status of cells and to adjust their metabolism to changing energetic conditions. When nutrients are available mTORC1 is dynamically recruited to the lysosomal surface where it becomes active and promotes cellular anabolic processes (Saxton & Sabatini, 2017).

Recent studies have provided compelling evidence that lysosomal biogenesis and autophagy are controlled by the master transcriptional regulator transcription factor EB (TFEB) (Sardiello *et al*, 2009; Settembre *et al*, 2011). In addition to TFEB, another member of the MiT-TFE family of transcription factors, TFE3, has a partially redundant function and is regulated in a similar manner (Martina *et al*, 2014; Raben & Puertollano, 2016).

TFEB subcellular localization and function is regulated by nutrient-induced mTORC1-mediated phosphorylation of specific serine residues (Settembre *et al*, 2012; Roczniak- Ferguson *et al*, 2012; Martina *et al*, 2012). On phosphorylation by mTORC1, TFEB is retained in the cytoplasm. A variety of stimuli leading to mTORC1 inactivation, such as starvation, induce TFEB dephosphorylation and nuclear translocation. Thus, the mTORC1-TFEB regulatory axis enables lysosomes to adapt their function to environmental cues, such as nutrient availability (Ballabio & Bonifacino, 2020). Several additional conditions, such as infection, inflammation, physical exercise, endoplasmic reticulum stress, and mitochondrial damage, also promote TFEB nuclear translocation, highlighting the complexity of TFEB regulation (reviewed in Puertollano *et al*, 2018; Cinque *et al*, 2015).

Recent findings have added further complexity to the mechanisms by which mTORC1 phosphorylates TFEB (Napolitano *et al*, 2020). Unlike other substrates of mTORC1, TFEB is known to interact with RagGTPases (Martina & Puertollano, 2013). Due to this interaction, TFEB phosphorylation occurs through an mTORC1 substrate-specific mechanism that is strictly dependent on the amino acid-induced activation of RagC and RagD GTPases but is insensitive to Rheb activity induced by growth factors. This allows mTORC1 activity to be differentially regulated by different stimuli (Napolitano *et al*, 2020). This substrate-specific regulation of TFEB by the mTORC1 pathway has a crucial role in Birt–Hogg–Dubé syndrome, a disorder characterized by benign skin tumors, lung, and kidney cysts and renal cell carcinoma (Kauffman *et al*, 2014; Calcagnì, *et al*, 2016) and caused by mutations in the lysosomal RagC/D activator folliculin (FLCN) (Napolitano *et al*, 2020).

Lysosomes are emerging as calcium (Ca^{2+}) storage organelles. The concentration of free Ca^{2+} within the lysosome is around 500 μM, and therefore comparable to endoplasmic reticulum (ER) Ca^{2+} levels (Christensen *et al*, 2002). Ca^{2+} channels, such as transient receptor potential mucolipin-1 (TRPML-1, Mucolipin 1, MCOLN1) and the two-pore channel (TPC), reside on the lysosomal

membrane and have been shown to mediate local Ca^{2+} signals from intracellular compartments (e.g., mitochondria) (Xu *et al*, 2015; Xu & Ren, 2015). Lysosomal Ca^{2+} signaling participates in multiple cellular processes such as lysosomal acidification, the fusion of lysosomes with other cellular organelles, membrane trafficking and repair, autophagy, and formation of contact sites between the lysosome and the endoplasmic reticulum (Kilpatrick *et al*, 2013; Lloyd-Evans & Waller-Evans, 2019). Furthermore, lysosomal Ca^{2+} signaling is involved in the regulation of lysosomal biogenesis and autophagy through the activation of TFEB. Upon starvation, lysosomal Ca^{2+} release through TRPML1 activates the Ca^{2+}-dependent serine/threonine phosphatase calcineurin (CaN), which binds and dephosphorylates TFEB, thus promoting its nuclear translocation (Medina *et al*, 2015). TRPML1 also induces autophagic vesicle biogenesis through the generation of phosphatidylinositol 3-phosphate (PI3P) and the recruitment of essential PI3P-binding proteins to the nascent phagophore in a TFEB-independent manner (Scotto-Rosato *et al*, 2019).

The nosography of LSDs

LSDs are multisystem disorders that are associated with a broad range of clinical manifestations affecting multiple organs and systems and causing visceral, ocular, hematologic, skeletal, and neurological signs. These manifestations are often highly debilitating, causing progressive physical and neurological disabilities.

In general, LSD presentations show broad variability (Beck, 2001), ranging from early-onset (in some cases neonatal), severe clinical forms that often result in premature death of patients, to late-onset, attenuated phenotypes that have a lesser impact on patient health and lifespan. Albeit individually rare, their cumulative incidence is estimated in approximately 1 in 5,000–7,500 births, with higher rates in specific populations. It is noteworthy that newborn screening programs for LSDs, now active in some countries, may in the future significantly change these estimates and will likely provide a more precise figure of LSD incidence (Spada *et al*, 2006; Hopkins *et al*, 2018; Wasserstein *et al*, 2019).

The nosography of LSDs has evolved over time, reflecting the advancements in the knowledge of lysosomal function and the cellular consequences of its dysfunction. The traditional classification based on the classes of stored substrates (glycosaminoglycans in the mucopolysaccharidoses, glycosphingolipids in the glycosphingolipidoses, glycoproteins in the oligosaccharidosis, etc) largely reflects the vision of lysosomes as catabolic organelles and is centered on the disease biochemistry. Accurate and exhaustive information on LSD classification and nosography can be found elsewhere (Platt *et al*, 2018).

With the improved knowledge on the molecular and cellular bases of LSDs, an alternative way of classifying LSDs has been proposed, based on the process that is defective in the biogenesis of lysosomal enzymes, rather than on the stored substrate (Platt, 2018). The majority of these disorders is due to deficiencies of soluble hydrolases that are involved in the sequential degradation of a specific substrate. Other disorders are due to deficiencies in upstream processes, such as post-translational modifications (multiple sulfatase deficiency, MSD, due to the lack of an enzyme converting a cysteine into a formylglycine residue in the catalytic site of sulfatases) (Cosma *et al*, 2002; Dierks *et al*, 2002), or to defective sorting of lysosomal enzymes to lysosomes (mucolipidoses—ML—II and III, with deficient generation of mannose-6-phosphate) (Hickman & Neufeld, 1972). Others are due to mutations of non-enzymatic activator proteins (saposin activator protein, SAP, deficiencies) (Tamargo *et al*, 2012), of solute carriers (cystinosis, infantile sialic acid storage disease) (Gahl *et al*, 1982; Verheijen *et al*, 1999) and other lysosomal membrane proteins (Danon disease, due to LAMP2 defective function) (Nishino *et al*, 2000; Tanaka *et al*, 2000), or are the consequence of defects in assembly and stability of multienzymatic complexes (galactosialidosis, due to cathepsin A deficiency) (d'Azzo *et al*, 1982).

New technologies and cellular modeling to study lysosomal function in health and disease

Novel technologies have had significant impacts on the characterization of lysosome biology, the development of diagnostic tools for patients with a suspicion of LSD, and the identification and validation of new therapeutic targets (Fig 2) (Table 1). In several cases, these approaches are based on high-throughput techniques combined with bioinformatic analysis of a large body of information (metabolomic, genomic, proteomic approaches). Novel approaches also include automated robotic-based, miniaturized or cell-based procedures (high-content imaging) as well as innovative techniques that allow for manipulation of genetic information and generation of *in vitro* and *in vivo* models of disease.

Genomic techniques and next-generation sequencing (NGS)

A major advancement, particularly in the diagnostic approach to LSDs, was introduced by NGS, a powerful diagnostic tool based on technological platforms that allow sequencing of millions of small fragments of DNA in parallel. This technology has been used both through a targeted strategy with gene panels and by untargeted approaches based on whole-exome sequencing.

Given the difficulties in the diagnostic work-up, and due to overlapping clinical phenotypes in LSDs, patients with a clinical suspicion of these disorders are excellent candidates for the application of an NGS-based diagnosis. Before the introduction of NGS, the traditional approach for the diagnosis of LSDs was based on a step-by-step, progressive process starting with physical examination, proceeding to metabolite identification in biological fluids, and leading to the exact diagnosis through the demonstration of an enzymatic deficiency (or the deficiency of a lysosomal function) and the identification of mutations in a specific gene (Winchester, 2014).

NGS-based approaches are substantially changing this stepwise process. The molecular analysis and search for mutations in LSD-related genes can be performed immediately after the clinical suspicion of LSD, whereas the functional analysis of the deficient enzyme (or a non-enzymatic protein) offers a complementary approach to definitively confirm disease diagnoses. Such a diagnostic process may prove to be cost-effective and by-pass the need for multiple biochemical analyses or repeated hospital admissions. Some examples of this strategy are already available in the literature, with the development of a gene panel specific for 891 genes involved in the ALP function (Di Fruscio *et al*, 2015), or the identification of Pompe disease patients in cohorts of unidentified limb-girdle muscular dystrophies (Savarese *et al*, 2018).

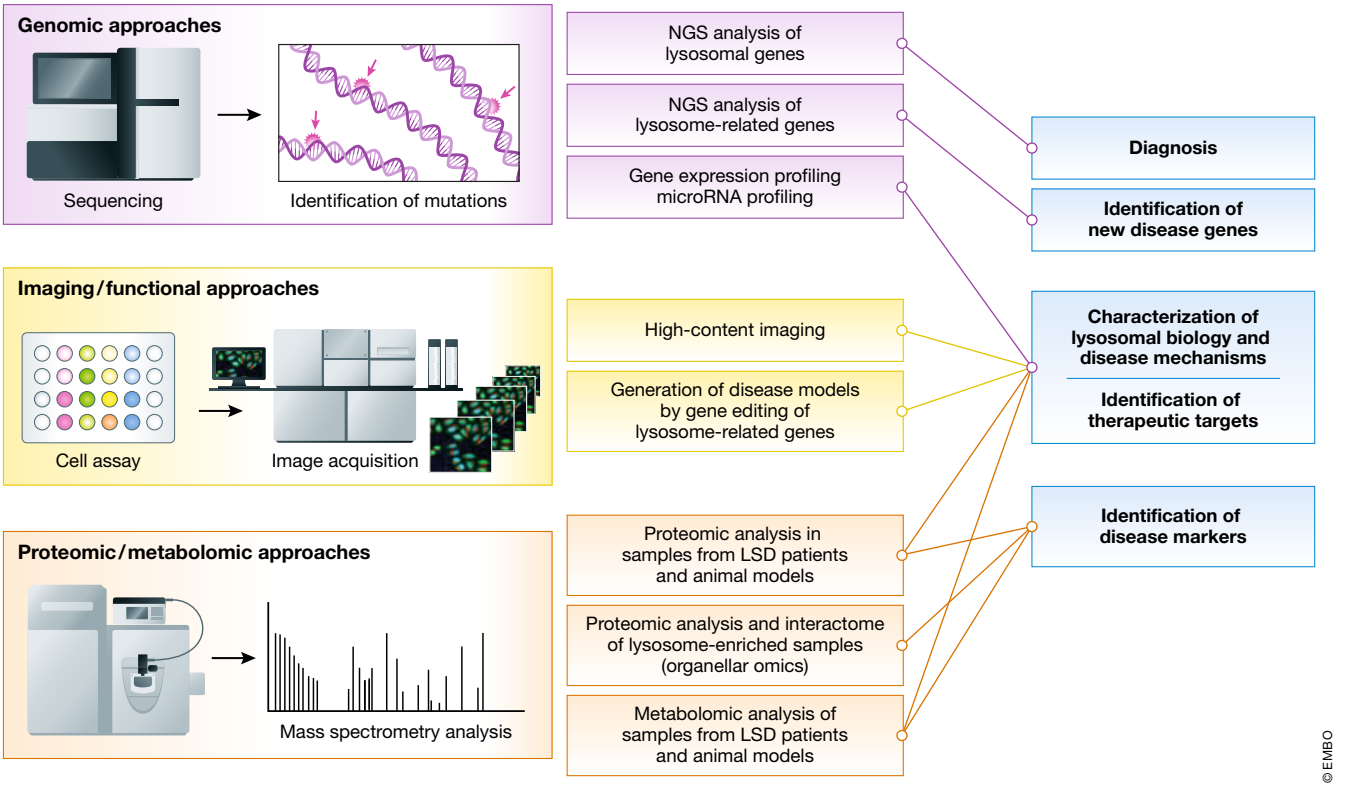

Figure 2. New technologies to study lysosomal function and biology.

New technologies, in most cases based on high-throughput techniques combined with bioinformatic analysis, have been exploited for the diagnostic work-up in patients with a suspected LSD, for the identification of new disease genes, for the search of disease biomarkers, for the characterization of lysosome biology and disease mechanisms, and for the identification and validation of new therapeutic targets.

NGS-based analysis also has the potential to identify new genes involved in lysosomal disorders, thus expanding the list of LSDs. Indeed, two novel disorders associated with lysosomal abnormalities and impaired vesicle trafficking were recently recognized through whole-exome sequencing. Mucopolysaccharidosis plus (MPS-plus), characterized by typical manifestations of mucopolysaccharidoses such as coarse facial features, skeletal abnormalities, hepatosplenomegaly, respiratory problems, and by remarkable levels of glycosaminoglycans excretion in urines (Kondo *et al*, 2017), was associated with mutations in the *VPS33A* gene (Pavlova *et al*, 2019). Loss-of-function *VPS16* gene mutations were found in patients with an early-onset dystonia and with ultrastructural lysosomal abnormalities (Steel *et al*, 2020). Both *VPS33A* and *VPS16* genes encode for subunits of the homotypic fusion and vacuole protein sorting (HOPS) complex that is essential for lysosome fusion with endosomes and autophagosomes (Wartosch *et al*, 2015).

Genomic approaches may also contribute to the understanding of the complexity and clinical variability of LSDs. The application of these approaches to the study of LSDs is a vast and rapidly expanding field that has been comprehensively reviewed in recent years (Hassan *et al*, 2017; Davidson *et al*, 2018).

Genome-wide association studies have been performed to identify modifiers in some LSDs, for example, Gaucher disease and Pompe disease. In Gaucher disease, single nucleotide polymorphisms (SNPs) within the *CLN8* gene locus were in linkage disequilibrium and associated with disease severity, possibly regulating sphingolipid sensing and/or in glycosphingolipid trafficking (Zhang *et al*, 2012). In Pompe disease, a c.510C > T variant was identified as a genetic modifier in late-onset patients. This variant negatively influences pre-mRNA splicing in patients carrying the c.-32-13T > G mutation, with significant correlations with residual alpha-glucosidase activity and may be predictive of clinical course and outcome in late-onset patients (Bergsma *et al*, 2019).

Transcriptomic analysis has been performed in several types of LSDs, such as Niemann-Pick disease type C (Martin *et al*, 2019), mucopolysaccharidoses (Salvalaio *et al*, 2017; Peck *et al*, 2019), progranulin deficiency (Evers *et al*, 2017), Pompe disease (Turner *et al*, 2016), and Gaucher disease (Dasgupta *et al*, 2013), mucolipidosis type IV (Cougnoux *et al*, 2019). Although the animal models and the tissues differed in these analyses, it was possible to recognize a few common patterns in some diseases. In mucolipidosis type IV mouse microglia, the mixed neuroprotective/neurotoxic expression pattern showed similarities with that observed in Niemann-Pick disease type C1 (Cougnoux *et al*, 2019). Of note, the changes observed in mucolipidosis type IV microglia overlapped with alterations found in common neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases. The analysis

Table 1. Examples of application of novel technologies for LSDs.

Technology	Applications	Examples of successful applications
Genomic sequencing	• Diagnosis of LSD patients and identification of mutations of known genes • Identification of mutations in genes not associated with LSD	Identification of new LSDs caused by mutation of the *VPS33A* (Pavlova *et al*, 2019) and *VPS16* (Steel *et al*, 2020) genes
Transcriptomic analysis	• Information on pathways involved in disease pathophysiology • Response to environmental conditions /pharmacological manipulations	Similarities between the microglia expression profiles of LSDs (mucolipidosis type IV mouse and Niemann-Pick disease type C1) with common neurodegenerative disorders (Cougnoux *et al*, 2019)
Genome-wide association studies	• Identification of modifying factors • Information on disease pathophysiology	Identification of a c.510C > T variant that may be predictive of clinical course and outcome in late-onset Pompe disease patients (Bergsma *et al*, 2019)
microRNA sequencing	• Identification of disease biomarkers that correlate with disease severity and assist in monitoring disease progression and efficacy of therapies • Identification of pathways involved in disease pathophysiology	Identification of differentially expressed microRNAs potentially predictive of disease severity in Pompe disease (Tarallo *et al*, 2019)
Biochemical and metabolomic analyses	• Support and validation of diagnosis • Identification of disease biomarkers that correlate with disease severity, monitoring disease progression, monitoring efficacy of therapies • Newborn screening • Identification of pathways involved in disease pathophysiology	Identification of disease biomarkers for several LSDs (Boutin & Auray-Blais, 2015; Reunert *et al*, 2015; Polo *et al*, 2019) Development of methods for simultaneous detection of multiple enzyme activities in dried blood spots suitable for newborn screening programs for several LSDs (Anderson, 2018; Donati *et al*, 2018; Kumar *et al*, 2019; Lukacs *et al*, 2019; Scott *et al*, 2020)
Cell-based assays and high-content imaging technologies	• Identification of pathways involved in disease pathophysiology • Screening for correctors and therapeutic agents	Development of multiplex staining assays that allow screening of FDA-approved compounds and identification of correctors for cellular phenotypes of LSDs (Pipalia *et al*, 2006; Pugach *et al*, 2018)
Targeted gene knock-out and genome editing—iPSc	• Identification of pathways involved in disease pathophysiology • Screening and validation of therapeutic agents • Gene editing of mutant genes to correct disease-causing mutations	CRISPR-Cas9-mediated generation of knock-out models of LSDs, such as sphingolipidoses and Niemann-Pick disease type C (Santos & Amaral, 2019)
Organellar omics	• Information on lysosome biology • Identification of pathways involved in disease pathophysiology • Identification of disease biomarkers for correlations with disease severity, monitoring disease progression, monitoring efficacy of therapies	Identification of lysosomal proteome and interactome (Sleat *et al*, 2005; Abu-Remaileh *et al*, 2017; Thelen *et al*, 2017; Rabanal-Ruiz & Korolchuk, 2018)

of mid-cervical cord in the mouse model of Pompe disease showed up-regulation of pathways associated with cell death, proinflammatory signaling, and dysregulation of signal transduction pathways suggestive of impaired synaptic function and plasticity (Turner *et al*, 2016). Transcriptomic analysis in Gaucher disease mice revealed dysregulation of genes involved in cell growth and proliferation, cell cycle, heme metabolism, and mitochondrial dysfunction in liver, lung, and spleen (Dasgupta *et al*, 2013).

Albeit interesting, these data are largely affected by heterogeneity of the samples, indicating the need for integrated and systematic approaches in homogenous animal and cellular models.

In addition to disease mechanisms, NGS-based analysis also has the potential to identify novel disease biomarkers. This approach has been used in recent studies that identified microRNAs as markers of Pompe disease, with correlations between disease phenotype and severity, and possibly with the response to enzyme replacement therapy (Cammarata *et al*, 2018; Tarallo *et al*, 2019; Carrasco-Rozas *et al*, 2019). In Pompe disease, this analysis showed altered expression of microRNAs implicated in signaling pathways related to the pathophysiology of the disease like the mTOR and AMPK pathways, ubiquitin-mediated proteolysis, cardiac hypertrophy, muscle atrophy, and regeneration, regulation of stem cells pluripotency and myogenesis. One of the differentially expressed microRNAs, miR-133a, was identified as a potential marker of disease severity and response to therapy (Tarallo *et al*, 2019).

In Fabry disease, some of the dysregulated microRNAs are also related to the disease pathophysiology, such as miR-199a-5p and miR-126-3p that are known to be involved in endothelial dysfunction, and miR-423-5p and miR-451a that are involved in myocardial remodeling (Cammarata *et al*, 2018).

Biochemical, metabolomic analyses, and newborn screening for LSDs

New technologies for high-throughput analysis of multiple metabolites have also significantly contributed to the diagnostics and monitoring of LSDs. The availability of measurable and objective disease markers remains a major challenge for many LSDs.

Clinical measures are often non-specific (such as the 6-min walk test, respiratory function tests, quality of life assessments) or can be influenced by inter- and intra-investigator variance. Biochemical markers may complement these clinical measures and provide accessory, quantitative tools to follow disease course and patient response to therapies. The search for biochemical markers has benefited from a number of modern methodologies such as mass spectrometry or nuclear magnetic resonance-based approaches. Mass spectrometry has become the most widely used platform for inborn metabolic diseases because of its ability to analyze a wide range of molecules in different body fluids, with optimal dynamic range and great sensitivity (Costanzo *et al*, 2017). Metabolome analyses have been performed in some LSDs, such as some mucopolysaccharidoses (Fu *et al*, 2017; Tebani *et al*, 2019), Fabry disease (Boutin & Auray-Blais, 2015), Pompe disease (Sato *et al*, 2017), Niemann-Pick disease type C (Fan *et al*, 2013; Maekawa *et al*, 2015; Probert *et al*, 2017), neuronal ceroid lipofuscinoses (Sindelar *et al*, 2018), or in some sphingolipidoses (Polo *et al*, 2019).

This approach has allowed the identification of markers that may serve to facilitate early-stage diagnosis and monitoring of response to therapy, for example, galabiosyl ceramide analogs in Fabry disease (Boutin & Auray-Blais, 2015), oxysterols in Niemann-Pick disease type C2 (Reunert *et al*, 2015), and various sphingolipids in some sphingolipidoses (Reunert *et al*, 2015). However, some issues need to be addressed in these studies, such as those related to the heterogeneity of samples used for the analyses and the number of patients required to be recruited in order to satisfy statistical significance (Percival *et al*, 2020).

Tandem mass spectrometry and digital microfluidic fluorimetry (DMF-F) have found important applications in the context of neonatal screening programs (Burlina *et al*, 2018; Gelb *et al*, 2019).

The first trials of newborn screening for LSDs started about two decades ago with an immunoassay for the lysosomal marker lysosomal-associated membrane protein-1 (LAMP-1) in dried blood spots (Ranieri *et al*, 1999). Other approaches followed, such as a multiplex immune-quantification assay of lysosomal enzymes (Meikle *et al*, 2006), enzyme assays by DMF-F, and tandem mass spectrometry (Gelb *et al*, 2019). Methods to test lysosomal enzyme activities in dried blood spots suitable for newborn screening programs have been developed for Fabry disease, Gaucher disease, Krabbe disease, Niemann-Pick A/B, Pompe disease, and mucopolysaccharidoses, and for less prevalent disorders such as alpha-mannosidosis, alpha-fucosidosis, lysosomal acid lipase deficiency, and ceroid lipofuscinosis 1 and ceroid lipofuscinosis 2 (Anderson, 2018; Donati *et al*, 2018; Kumar *et al*, 2019; Lukacs *et al*, 2019; Scott *et al*, 2020). In most instances, these methods allow for simultaneous detection of multiple LSDs (Kumar *et al*, 2019; Lukacs *et al*, 2019; Hong *et al*, 2020).

These screening programs, already active in several countries (Schielen *et al*, 2017), have had a significant impact on the care of LSDs, allowing early diagnosis and timely access to therapies (Chien *et al*, 2009) and have changed the figure of LSD prevalence in different countries and populations (Spada *et al*, 2006).

Cell-based assays and high-content imaging technologies

Together with robotics, the development of cell-based assays in combination with high-content imaging technologies for screening has represented a major and fascinating advance for diseases that are associated with an evident cellular phenotype. Cell-based high-content imaging usually exploits automated microscopy and complex image algorithms to extract multidimensional information from hundreds of samples, such as cell morphology, fluorescence intensity, or distribution of fluorescent markers within cells (Bellomo *et al*, 2017). Results usually have very high statistical power, due to the high number of cells that can be analyzed allowing averaging of large amount of data. The major advantage, however, is represented by the possibility of multiplexing different assays simultaneously in integrated cell populations, cell subpopulations, individual cells, and subcellular structures within a given population (Zanella *et al*, 2010; Peravali *et al*, 2011). For these reasons, high-content imaging is currently used to study disease mechanisms by loss-of-function and gain-of-function studies, as well as in preclinical drug discovery including target identification, lead optimization, assay validation, or primary and secondary screenings (Bellomo *et al*, 2017).

Several studies substantiate the potential of cell-based screening approaches to identify candidate molecules for the treatment of LSDs, some of them exploiting high-content imaging assays. For example, lysotracker and filipin fluorescence staining assays, or multiplex staining with dual markers filipin and anti-LAMP1 have been exploited to screen FDA-approved compounds and identify correctors of Niemann-Pick disease type C1 (Pipalia et a, 2006; Pugach *et al*, 2018). One such drug is the antimicrobial alexidine dihydrochloride that appeared to promote increases in *NPC1* transcript and mature protein and to be a potent cholesterol-reducing drug. A cell-based assay was also developed to detect arylsulfatase A residual activity in cells from patients with metachromatic leukodystrophy (Geng *et al*, 2011). Furthermore, a cell-based assay was used to identify three compounds that enhance galactocerebrosidase activity (Jang *et al*, 2016). Another phenotypic-based approach was used to identify modulators of autophagy in a murine neuronal cell model of CLN3 disease and led to the identification of compounds that normalized lysosomal positioning and promote clearance of storage material (Petcherski *et al*, 2019).

In summary, together these data, albeit preliminarly, suggest that cell-based screening approaches may lead to the development of novel therapeutics for lysosomal storage diseases.

Targeted gene knock-out and genome editing techniques

Recently, the development of CRISPR/Cas9 approaches for genome editing and the technology for the generation of induced pluripotent stem cells (iPSCs) from skin or blood samples have opened a new era for the development of disease-relevant cellular models of genetic diseases. These tools have found particular application for the generation of drug-based screening systems and for the study of disease pathophysiology. iPSCs derived from patients are pluripotent and capable of differentiating into virtually any cell type, including disease-relevant neuronal subtypes (Khurana *et al*, 2015) that display major common features of LSD pathology such as autophagic, lysosomal maturation, and mitochondrial defects (Lojewski *et al*, 2014). However, a major inconvenience of patient-derived iPSCs is their genetic diversity. The use of (CRISPR)-Cas9

gene editing to introduce specific genetic changes into a parental pluripotent line allows the production of isogenic lines representing selected mutations of LSD genes that can more readily be compared (Chaterji *et al*, 2017). CRISPR-Cas9 is currently being exploited to generate knock-out models for the study of LSDs. An exhaustive review about modeling of both cellular and *in vivo* modeling of rare sphingolipidoses and Niemann-Pick disease type C, using this technology, has been published recently (Santos & Amaral, 2019).

Both CRISPR-Cas9- and iPSC-based approaches offer unprecedented opportunities for the generation of cellular models of LSDs. These can be used for the identification of new pathways involved in the physiology and pathogenesis of LSDs and for drug screenings to identify of new drugs for the therapy of these diseases. Additionally, CRISPR/Cas9 can be by itself an alternative therapeutic option for treating LSDs by correcting disease-causing mutations, both *in vitro* and *in vivo* (Schwank *et al*, 2013; Wu *et al*, 2013; Xie *et al*, 2014).

Targeted gene knock-out and knock-in technologies have found important applications in the generation of *in vivo* models of LSDs. Compared to *in vitro* systems, animal models have major advantages as they provide opportunities to study pharmacokinetics, bioavailability, toxicity of therapeutic agents, and to evaluate critical endpoints such as metabolic responses in organs and tissues, and functional measures (Moro & Hanna-Rose, 2020). Animal models have been developed for nearly all LSDs (Vaquer *et al*, 2013), the majority in small, prolific species such as mice and rats (Vaquer *et al*, 2013; Gurda & Vite, 2019). Knock-in animal models have peculiar advantages as they allow characterization of the phenotypic, pathologic, and functional consequences of specific mutations found in patients (Praggastis *et al*, 2015) or for testing the response of such mutations to experimental treatments (Khanna *et al*, 2010).

Organellar omics

The characterization of the lysosomal proteome has been exploited as an important tool for the understanding of lysosomal dysfunction in human disease and of LSD pathophysiology. Proteomic analysis of lysosomes is mainly based on tandem mass spectrometry and has already proven to be a suitable strategy for the analysis of proteins interactions (Sleat *et al*, 2005), for the identification of biomarkers (Cologna *et al*, 2012; Matafora *et al*, 2015), for the identification of the molecular bases of LSDs, such as CLN2 (Sleat *et al*, 1997) and NPC2 (Naureckiene *et al*, 2000), and for the identification of novel potential lysosomal membrane transporters (Chapel *et al*, 2013).

Proteome analyses in LSD animal models and in selected samples from patients have been performed for Krabbe disease (Pellegrini *et al*, 2019), mucolipidosis III (Di Lorenzo *et al*, 2018) ceroid lipofuscinoses (Sleat *et al*, 2019), Niemann-Pick type C1 (Pergande *et al*, 2019), in some mucopolysaccharidoses (Yuan *et al*, 2019), and others. In recent years, in light of the emerging role of lysosomes as signaling platforms controlling cellular metabolism, this approach has attracted renewed and growing interest. Depending on the studies, approximately 200 proteins have been identified, including *bona fide* lysosomal and lysosomal-associated proteins. Interestingly, most of the lysosomal-associated proteins correspond to members of the mTORC1 complex (Thelen *et al*, 2017; Rabanal-Ruiz & Korolchuk, 2018). Searching for lysosomal terms in gene ontology and protein databases resulted in the identification of at least 500 proteins (Mi *et al*, 2019; The UniProt Consortium, 2019).

However, this approach is associated with important challenges related to the limited sensitivity of technologies in detecting low abundance proteins in lysosomes, lack of spatial information about the localization of the identified proteins in whole-cell analysis, and interaction with other organelles. In recent years, methods to reduce the biomolecular complexity of a sample by isolation and purification of individual cellular organelles have been developed (Diettrich *et al*, 1998; Chen *et al*, 2005; Walker & Lloyd-Evans, 2015; Tharkeshwar *et al*, 2017). These methods include density gradient centrifugation of cellular or tissue homogenates, use of magnetic iron oxide (FeO)-coated high-molecular-weight dextran particles to purify lysosomes from mammalian cells (Diettrich *et al*, 1998; Chen *et al*, 2005), and delivery of superparamagnetic iron oxide nanoparticles (SPIONs) to lysosomes by endocytosis (Walker & Lloyd-Evans, 2015). In this regard, it is important to mention the importance of the protein localization database based on careful subcellular localization studies combined with MS analysis (Prolocate, http://prolocate.cabm.rutgers.edu). More recent approaches are based on immunoaffinity enrichment of lysosomes from cells expressing a lysosomal transmembrane protein (i.e., TMEM192) fused to three tandem human influenza virus hemagglutinin (HA) epitopes using an antibody against HA conjugated to magnetic beads (Abu-Remaileh *et al*, 2017; Wyant *et al*, 2018). This method is rapid at extracting highly pure lysosomes and may represent an important advance in the analysis of the lysosomal proteome and in the quantitative profiling of metabolites derived from the action of lysosomal enzymes or from the activity of lysosomal transporters under various cell states (Abu-Remaileh *et al*, 2017).

Changes in the amounts of specific metabolites within the lysosome might uncover novel aspects involved in the pathophysiology of complex LSD and lead to the identification of aberrant accumulation of specific cargo or metabolites that could be used as biomarkers to test the efficacy of novel therapies.

How defects of lysosomal functions lead to disease

Historically, the pathology and the clinical manifestations of lysosomal disorders have been considered as direct consequences of the storage of inert substrates in tissues.

Indeed, manifestations such as visceromegaly, skin thickness, skeletal dysmorphisms, and ocular signs (corneal opacities, cherry-red spot) might easily be viewed as the results of excessive undegraded substrates in cells and extracellular matrix.

This concept has been questioned by the new vision of lysosomal functions. Given the central role of lysosomes in cellular homeostasis and metabolism, it has been speculated that storage is just the "instigator" of a number of secondary events (Clarke, 2011) and that accumulation of undegraded substrates is able to prime complex pathogenetic cascades that are in fact responsible for LSD manifestations. Multiple and diverse events are now emerging as players in the pathogenesis of LSDs. Specifically, these events include storage of secondary substrates unrelated to the defective enzyme, abnormal composition of membranes and aberrant fusion and intracellular trafficking of vesicles, altered autophagic flux and accumulation of autophagic substrates, dysregulation of signaling

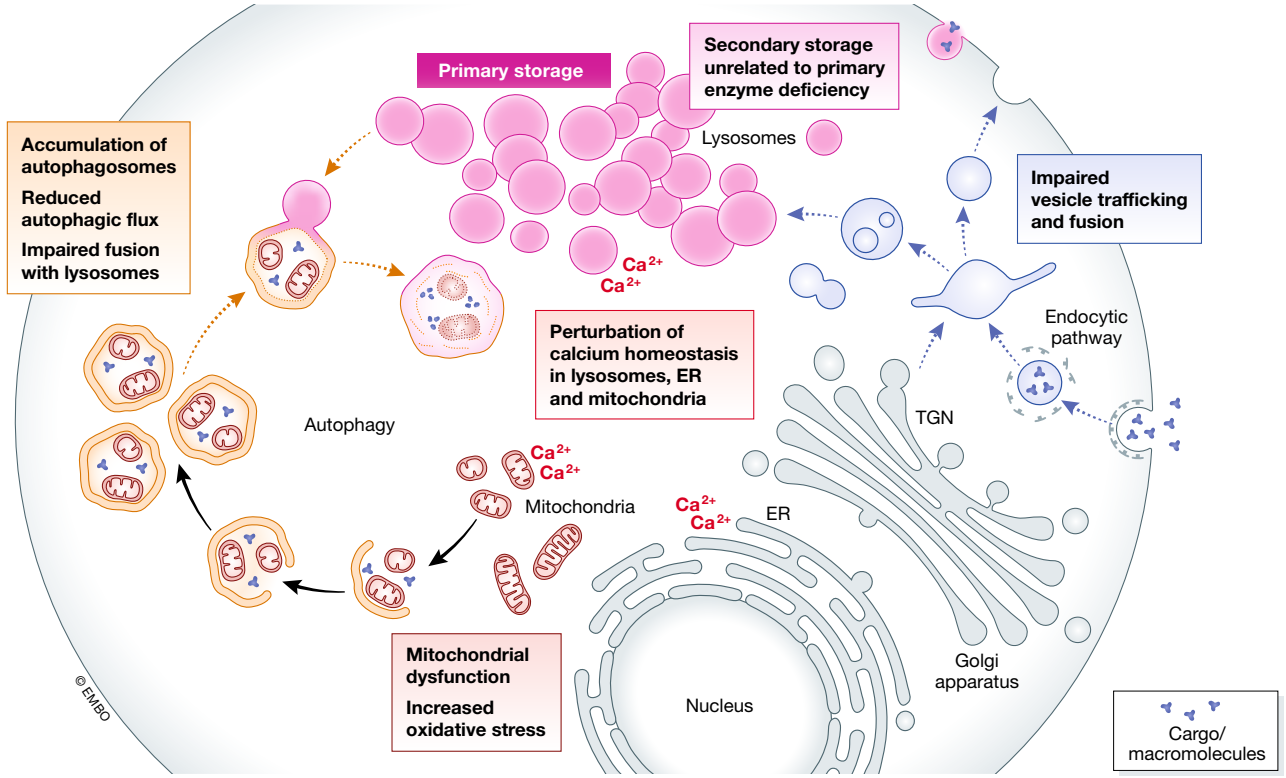

Figure 3. The mechanisms involved in the pathophysiology of LSD.

The accumulation of undegraded substrates triggers multiple events that are currently emerging as important players in the pathogenesis of LSDs. These events include storage of secondary substrates unrelated to the defective enzyme; abnormal composition of membranes and aberrant fusion and intracellular trafficking of vesicles; impairment of autophagy; perturbation of calcium homeostasis; and mitochondrial dysfunction and oxidative stress. In addition to the events shown in the figure, in several LSDs storage triggers dysregulation of signaling pathways and activation of inflammation.

pathways and activation of inflammation, abnormalities of calcium homeostasis, mitochondrial dysfunction, and oxidative stress (Ballabio & Gieselmann, 2009; Platt *et al*, 2018) (Fig 3).

Secondary storage

Secondary storage of unrelated and heterogeneous substrates has been extensively documented in several LSDs. For example, in mucopolysaccharidoses types I, II, IIIA, VI, and VII, characterized by primary storage of glycosaminoglycans, biochemical analysis of brain has shown that gangliosides GM2 and GM3 are also consistently and substantially elevated (Walkley & Vanier, 2009). Accumulation of GM2 and GM3 gangliosides has also been documented in a variety of other LSDs, including Niemann-Pick type A and type C1 (Zervas, *et al*, 2001), mucolipidosis type IV (Micsenyi *et al*, 2009) neuronal ceroid lipofuscinoses (Jabs *et al*, 2008), and alpha-mannosidosis (Goodman *et al*, 1991).

Secondary storage is thought to have a substantial role in the pathophysiology of LSDs. This idea is supported by the finding that secondary substrates localize in the brain areas that are most affected by the disease pathology (Tobias *et al*, 2019; Viana, *et al*, 2020) and that depletion of secondary substrates in experimental conditions (e.g., GM3 in the Niemann-Pick type C1 mouse model) results in amelioration of neuropathology and disease manifestations (Lee *et al*, 2014). Secondary storage may also impair vesicle trafficking. Cholesterol and other lipids, for example, have been shown to impair the endo-lysosomal system (Sobo *et al*, 2007; Walkley & Vanier, 2009), and the function of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Fraldi *et al*, 2010; Sambri *et al*, 2017), which are critical for the fusion of cellular membranes (Lang *et al*, 2001).

These effects are not only limited to lysosomes but also implicated in the accumulation in other compartments of toxic storage materials, including multiple aggregate-prone proteins, α-synuclein, prion protein, Tau, amyloid β, and damaged mitochondria (Fraldi *et al*, 2016) that are known to be associated with common neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's disease. Alpha-synuclein accumulation has been found in several LSDs (Settembre *et al*, 2008a; Shachar *et al*, 2011; Di Malta *et al*, 2012) and suggests a link between alpha-synuclein aggregation toxicity and neurodegeneration in LSDs. Extensive work has been done about beta-glucocerebrosidase deficiency as a risk factor for the development of Parkinsonism (Aharon-Peretz *et al*, 2004; Gan-Or *et al*, 2008; Blanz & Saftig, 2016).

Impairment of autophagy

As lysosomes are the terminal compartment of the ALP, a general impairment of this pathway and of its critical functions in cell homeostasis is an obvious and expected finding in LSDs. A block of

autophagy was initially recognized in a few of these disorders, namely in mucopolysaccharidosis type IIIA and in MSD (Settembre *et al*, 2008a), as well as in Pompe disease in which the accumulation of large pools of autophagic debris is a typical and consistent feature of skeletal muscle pathology (Fukuda *et al*, 2006a; Shea & Raben, 2009) and appears to be associated with dysregulation of mTORC1 and AMPK signaling (Lim *et al*, 2017).

Autophagic vesicle accumulation, together with increased poly-ubiquitinated proteins and dysfunctional mitochondria, has now been reported also in several other LSDs, such as some mucopolysaccharidoses, sphingolipidoses (Gaucher and Fabry disease, Niemann-Pick type C), mucolipidoses (type II, III and IV), Danon disease, and some neuronal ceroid lipofuscinoses (CLN 3, 10) (Liebermann *et al*, 2012).

The impairment of autophagy has important and deleterious consequences and is thought to contribute substantially to LSD pathophysiology. In Pompe disease, for example, the presence of autophagic accumulation was shown to impair the contractile function of muscles (Drost *et al*, 2005) and to affect the trafficking of recombinant enzymes used for enzyme replacement therapy (Fukuda *et al*, 2006b).

In neurons, a dysfunction of the ALP is thought to be involved in the pathophysiology of neurodegeneration (Hara *et al*, 2006; Komatsu *et al*, 2006; Monaco *et al*, 2020). The impairment of the ALP has also been shown to affect extracellular matrix formation and skeletal development and growth in chondrocytes from the mouse model of MPS VII (Bartolomeo *et al*, 2017; Settembre *et al*, 2018).

Mitochondrial dysfunction

One of the primary functions of autophagy is to execute mitochondrial turnover (Plotegher & Duchen, 2017; Wang & Wang, 2019). Thus, it is not surprising that mitochondrial dysfunction is emerging as an important player in the pathophysiology of LSDs. Substantial evidence supports the existence of a crosstalk and reciprocal functional relationships between mitochondria and lysosomes (Pshezhetsky, 2015). While defective autophagy affects mitochondrial quality control pathways and causes accumulation of damaged mitochondria, in turn mitochondrial dysfunction can impair lysosomal functions, such as acidification by the acidic pump V-ATPase that relies on the ATP generated by mitochondria (Stepien *et al*, 2020).

Perturbations in mitochondrial function and homeostasis have been recognized in several LSDs, including sphingolipidoses (Gaucher disease, Niemann-Pick disease type C, Krabbe disease), gangliosidoses, some mucopolysaccharidoses, multiple sulfatase deficiency, and neuronal ceroid lipofuscinoses (Plotegher & Duchen, 2017; Stepien *et al*, 2020) and have been proposed as one of the mechanisms underlying neurodegeneration (Martins *et al*, 2015; Saffari *et al*, 2017; Annunziata *et al*, 2018).

Multiple mitochondrial defects were found in the mouse model of Pompe disease, including mitochondrial calcium excess, increased reactive oxygen species, decreased mitochondrial membrane potential, and decreased oxygen consumption and ATP production (Lim *et al*, 2015). Increased oxidative stress, elevation of reactive oxygen species (ROS), and enhanced susceptibility of cells to mitochondria-mediated apoptotic insults are obvious consequences of defects in mitophagy and mitochondrial dysfunction (Filomeni *et al*, 2015). Oxidative stress was observed in animal models of mucopolysaccharidoses type IIIB (Villani *et al*, 2009), type I (Donida *et al*, 2015), and type IIIA (Arfi *et al*, 2011) and in blood samples from patients affected by mucopolysaccharidoses types I (Pereira *et al*, 2008) and type II (Filippon *et al*, 2011).

Alteration of signaling pathways and inflammation

Non-physiologic activation of signal transduction pathways by storage compounds is another consequence of storage in LSDs. Stored materials may interfere with normal ligand–receptor interactions, modify receptor responses, influence internalization and recycling of receptors, and lead to altered activation of signaling pathways involved in cellular transport and vesicle trafficking, calcium homeostasis, oxidative stress, morphogen signaling, inflammatory and innate immune responses, and cell death (von Zastrow & Sorkin, 2007; Ballabio & Gieselman, 2009; Fiorenza *et al*, 2018; Platt *et al*, 2018).

Neuroinflammation and bone involvement are paradigm examples of manifestations related to altered signaling. Neuroinflammation has been reported in a large variety of LSDs, such as mucopolysaccharidoses (Zalfa *et al*, 2016; Viana *et al*, 2020; Heon-Roberts *et al*, 2020), sphingolipidoses (Potter & Petryniak, 2016; Fiorenza *et al*, 2018; Cougnoux *et al*, 2019), neuronal ceroid lipofuscinoses (Groh *et al*, 2016), and gangliosidoses (Utz *et al*, 2015). In some mucopolysaccharidoses, it has been shown that structurally anomalous glycosaminoglycans may mimic lipopolysaccharide, an endotoxin of gram-negative bacteria, and activate the Toll-like receptor 4 (TLR4) innate immune responses. As a consequence of TLR4 activation, secretion of proinflammatory cytokines increases together with the activation of tumor necrosis factor (TNF)-alpha (Simonaro *et al*, 2010; Parker & Bigger, 2019). Furthermore, the activation of an atypical pattern of interferon downstream signaling, involving both interferon (IFN)-gamma- and IFN-alpha-responsive genes, was detected in the cerebellum of the Niemann-Pick disease type C1 mouse model, resulting in the elevation of IFN-gamma-responsive cytokines (Shin *et al*, 2019).

Interestingly, aberrant morphogen signaling has emerged as a possible mechanism that may explain some clinical features of LSDs, such as skeletal dysmorphisms, traditionally viewed as a direct consequence of substrate accumulation (Fiorenza *et al*, 2018). For example, excess of accumulated glycosaminoglycans and defective proteoglycan desulfation have been shown to alter fibroblast growth factors-2 (FGF2)-heparan sulfate interactions and the FGF2 signaling pathway in a murine model of MSD (Settembre *et al*, 2008b), and bone morphogenetic protein (BMP)-4 signaling activity in mucopolysaccharidosis type I cells (Pan *et al*, 2005).

Altered FGF2 and Indian hedgehog distribution and impaired FGF2 signaling have been observed in growth plates from mucopolysaccharidosis type I mice (Kingma *et al*, 2016). In a zebrafish model of mucopolysaccharidosis type II, perturbations of glycosaminoglycan catabolism were associated with aberrant distribution and signaling of morphogens, such as sonic hedgehog (Shh), dysregulation of the Shh and Wnt/β-catenin signaling, and aberrant heart development and atrioventricular valve formation (Costa *et al*, 2017).

Shh dysregulation, with severely disturbed subcellular localization of the Shh effectors Patched (Ptch) and Smoothened (Smo), and of ciliary proteins were found in Niemann-Pick disease type C1

mice. Dysregulation of Shh signaling has been associated with shortening of primary cilium length and reduction in ciliated cells in animal brains and was proposed as a mechanism underlying abnormal cerebellum morphogenesis in this mouse model (Canterini *et al*, 2017; Formichi *et al*, 2018).

Abnormalities of Ca^{2+} signaling are thought to play an important role in LSDs (Lloyd-Evans & Waller-Evans, 2019; Liu & Lieberman, 2019). The importance of this signaling pathway for LSD pathophysiology was revealed by mucolipidosis type IV. This disorder is due to mutations in the mucolipin 1 (*MCOLN1)* gene that encodes the lysosomal Ca^{2+}-releasing channel TRPML1. Mucolipidosis IV pathology is the consequence of defective TRPML1 function and aberrant Ca^{2+} signaling, and is characterized by impaired vesicle trafficking and extensive storage of granular material and lamellar and concentric bodies (LaPlante *et al*, 2004). Ca^{2+} abnormalities have also been found in other LSDs and thought to participate in disease pathophysiology. In Niemann-Pick type C1 mutant cells, for example, remarkable reduction in the acidic compartment calcium stores was observed, compared to wild-type cells, likely due to sphingosine storage that induces calcium depletion in lysosomes, possibly through an inhibitory effect on Na^{+}/Ca^{2+} exchangers (Lloyd-Evans *et al*, 2008; Lloyd-Evans & Platt, 2011).

Current and future therapies

Enzyme replacement therapy

Given that the majority of LSDs are due to the deficiency of a lysosomal hydrolase, the primary approach, and the first to be explored, was based on replacing the defective activity with a wild-type functional enzyme. This strategy was pursued through hematopoietic stem cell transplantation and enzyme replacement therapy (ERT), which are based on the concept that lysosomal enzymes can be taken up by cells and correctly delivered to lysosomes through the mannose-6-phosphate pathway.

After the pioneering studies in Gaucher disease (Barton *et al*, 1990; Barton *et al*, 1991) that demonstrated the feasibility and the efficacy of ERT in the non-neuronopathic forms of this disorder, this approach is currently considered the standard treatment for several LSDs, including the most prevalent of these disorders, such as Gaucher disease, Fabry disease, Pompe disease, some mucopolysaccharidoses, and a few ultra-rare disorders (e.g., lysosomal acid lipase deficiency, mucopolysaccharidosis type VII) and is in clinical development for others (Platt *et al*, 2018; Poswar *et al*, 2019). However, despite remarkable success in treating some aspects of LSDs, we have learned from the experience of many years that ERT has limitations. Recombinant enzymes are immunogenic and may induce immune responses, particularly in cross-reactive immunologic material (CRIM)-negative patients (Berrier *et al*, 2015). Development of antibodies against the therapeutic enzyme may impact on the efficacy of therapies or cause immune-mediated severe adverse reactions that require laborious and expensive protocols for induction of immune tolerance (Desai *et al*, 2019). ERT is based on periodic infusions, often requiring the use of intra-venous devices that are associated with risk of life-threatening infections. Thus, the impact on the quality of life of patients and their caregivers is substantial (Wyatt *et al*, 2012; Platt, 2018). In addition, costs of therapies are high and represent an economical burden for healthcare systems (Wyatt *et al*, 2012).

An additional and major issue is the insufficient biodistribution of the recombinant enzymes used for ERT, leading to inability to reach therapeutic concentrations in specific tissues. Recombinant enzymes are large molecules, often unable to cross anatomical and functional barriers. In several LSDs, the main target tissues, where the correction of enzyme activity is required to clear storage and improve pathology, are in fact the most difficult to reach (van Gelder *et al*, 2012). For these reasons, second-generation recombinant enzymes with improved targeting properties or pharmacodynamics are currently under development, for example, avalglucosidase-apha, a glycoengineered GAA, for the treatment of Pompe disease (Pena *et al*, 2019; Xu *et al*, 2019) and pegunigalsidase alfa, a PEGylated covalently cross-linked alpha-galactosidase A, for the treatment of Fabry disease (Schiffmann *et al*, 2019).

The inability of recombinant enzymes to cross the blood–brain barrier has major clinical relevance, as the majority of LSDs can be associated with neurological manifestations. Intrathecal or intraventricular delivery of enzyme has been proposed for some mucopolysaccharidoses (Giugliani *et al*, 2018), metachromatic leukodystrophy (I Dali *et al*, 2020), and is now an approved treatment for neuronal ceroid lipofuscinosis 2, in which intraventricular infusions of cerliponase-alpha reduced disease progression (Schulz *et al*, 2018). The use of chimeric lysosomal enzymes, in which the therapeutic enzyme is conjugated with different peptides or antibody components that exploit interactions with specific receptors and mediate transport across the blood–brain barrier, is also a strategy that is currently being investigated (Sonoda *et al*, 2018; Pardridge *et al*, 2018; Yogalingam *et al*, 2019).

Pharmacological therapy

Indeed, LSDs have proven to be an extraordinary field of investigation with the development of multiple and diverse therapeutic strategies that hit different targets in the pathogenetic cascade of these disorders (Cox, 2015).

One of these strategies was aimed to restore the equilibrium of the so-called "storage equation" (i.e., the balance between the amount of substrate that is delivered to lysosomes for degradation, and the amount of enzymes that are involved in its breakdown) by reducing flux of substrates to lysosomes with small-molecule inhibitors of substrate synthesis (substrate reduction therapy—SRT). The first of such compounds to be used was the imino sugar Miglustat (N-butyl-deoxynojirimicin), a reversible inhibitor of glucosylceramide synthase, that catalyzes the formation of glucocerebroside and thereby initiates the glycosphingolipid biosynthetic pathway (Pastores & Barnett, 2003). Miglustat showed efficacy in correcting some clinical and biochemical parameters in Gaucher disease patients (Cox, 2005; Charrow & Scott, 2015). For its effect on this early step of the glycosphingolipid biosynthetic pathway, Miglustat has also been proposed for the treatment of GM1 and GM2 gangliosidoses, with a slowed disease progression (Poswar *et al*, 2019; Fischetto *et al*, 2020), and is approved for the treatment Niemann-Pick disease type, in which gangliosides GM2, GM3 gangliosides, and other glycosphingolipids play a role in the pathogenesis of neurological manifestations (Zervas *et al*, 2001). Other substrate-reducing molecules are now in clinical development or approved for the treatment of Gaucher and Fabry disease, such as eliglustat tartrate, venglustat,

and lucerastat (Felis *et al*, 2019; Poswar *et al*, 2019). This approach is attractive, as substrate-reducing drugs are small molecules that reach therapeutic concentrations in tissues, including those that are difficult to reach by ERT, and can be taken orally, with minimal impact on patient quality of life.

Newer approaches, based on different strategies, are now in a phase of advanced development. In most cases, they are also aimed at increasing or replacing the defective enzyme activity. Pharmacological chaperone therapy (PCT) is based on the use of small-molecule drugs that enhance the stability of mutant enzyme proteins with residual activity, through specific non-covalent interactions with the target enzymes, thus favoring their trafficking to lysosomes (Parenti *et al*, 2015).

PCT is now approved for the treatment of patients affected by Fabry disease. Migalastat (1-deoxygalactonojirimycin), an active site-directed imino sugar reversible inhibitor of alpha-galactosidase A, was shown to paradoxically rescue the activity and the stability of this enzyme in cells from Fabry disease patients, opening the way to further development of this approach (Fan *et al*, 1999). This compound has shown clinical efficacy on different clinical manifestations of Fabry disease (Germain *et al*, 2016; Hughes *et al*, 2017; Lenders *et al*, 2020) and is now approved for the treatment of patients with amenable *GLA* gene mutations. PCT has evident advantages compared with ERT, as chaperones are small-molecule drugs that can be taken orally by patients and are expected to penetrate across membranes and physiological barriers, thus reaching therapeutic concentrations in multiple tissues. On the other hand, a major limitation of this approach is the possibility to treat only patients carrying specific mutations (Benjamin *et al*, 2017). Concerns on the use of PCT have also been raised as chaperones interact with the catalytic sites and are thus reversible competitive inhibitors of their target enzymes. For the treatment of Fabry disease, this risk has been eluded using a protocol based on discontinuous, every other day administration (Germain *et al*, 2016), taking advantage of the short half-life of the drug compared with that of alpha-galactosidase A. New, allosteric drugs that interact with non-catalytic domains of the enzyme and are thus non-inhibitory may represent an alternative strategy to minimize the risk of unwanted enzyme inhibition (Porto *et al*, 2012; Parenti *et al*, 2015a).

The use of proteostasis modulators has also been proposed to rescue mutant, unstable lysosomal enzymes (Mu *et al*, 2008; Fog *et al*, 2018; Seemann *et al*, 2020). However, the drugs used for this approach are non-specific and may be associated with significant adverse effects.

The demonstration of a synergy between PCT and ERT attracted further interest on this approach. This effect was first demonstrated *in vitro* and *in vivo* in cells from patients with Pompe disease and in the murine model of this disease (Porto *et al*, 2009; Khanna *et al*, 2012). This synergy was translated into clinical trials (Parenti *et al*, 2014; Kishnani *et al*, 2017) and is now under further clinical development (Data ref: ClinicalTrials.gov NCT04138277). The advantage of this synergy, compared with the "traditional" use of chaperones, is that the effect of the drug is directed toward the recombinant enzyme used for ERT, and not to the endogenous mutant enzyme. Thus, the effect of chaperones with this approach is mutation-independent and can in principle be extended to all patients on ERT. Also, the administration of the chaperone is limited to the time of the ERT infusion (e.g., every other week in Pompe disease), with less risk of undesired effects of the drug.

Gene therapy

Gene therapy holds great promise and is under advanced clinical development for several LSDs. The approaches used so far for gene therapy of LSDs are based both on the use of adeno-associated viral (AAV) vectors *in vivo* and of lentiviral vectors *ex vivo*. AAV-mediated *in vivo* gene transfer is based on injection of a vector carrying the transgene under the control of ubiquitous or organ-specific promoters. *Ex vivo* gene therapy is based on the correction of patient's cells, such as hematopoietic stem cells, followed by genetic modification *in vitro* and re-implantation in the patients of the modified cells. Both approaches imply both local correction of a target tissue/organ and cross-correction of distant tissues/organs by secreted enzymes that are internalized through the mannose-6-phosphate receptor pathway (Sands & Davidson, 2006).

Quite encouraging results have been obtained using the *ex vivo* approach in metachromatic leukodystrophy (Biffi *et al*, 2013; Sessa *et al*, 2016). Patients treated early showed improved course or complete prevention of disease manifestations, associated with improved brain MRI scores. *In vivo* AAV-mediated gene therapy studies have been completed (Corti *et al*, 2017) or are in progress.

However, like for ERT, important challenges remain to be faced also by gene therapy, particularly the need for sustained expression of the therapeutic enzyme, and the need for correction of neuropathology. To address this latter issue, studies aimed at correcting brain involvement through direct intraparenchymal or intrathecal vector administration have been performed for the treatment of mucopolysaccharidose types IIIA and IIIB (Tardieu *et al*, 2014; Tardieu *et al*, 2017).

Other approaches based on gene editing for mucopolysaccharidosis types I and II (Data ref: ClinicalTrials.gov NCT03041324), inhibition of nonsense-mediated decay and translational read-through (Banning *et al*, 2018), and messenger RNA (mRNA) therapy (an approach based on biosynthetic mRNA transcripts to drive the synthesis of therapeutic proteins) (Zhu *et al*, 2019) are under evaluation.

Other strategies that are currently under investigation are based on the use of antisense oligonucleotides that allow for rescue of the normal splicing of transcripts. For example, this approach appears to particularly attractive for the treatment of late-onset Pompe disease patients, in which a c.-32-13T > C mutation causes aberrant splicing and exon 2 partial or complete skipping with reduced synthesis of normal mRNA. This variant is highly prevalent (40–70% of alleles). Thus, a therapy for this mutation would have the advantage of being effective in a large fraction of patients. The characterization of splicing regulatory elements in GAA intron 1 and exon 2 and of the effects of the c.510C > T variant that modulates the effects of the c.-32-13T > C mutation (Bergsma *et al*, 2019) helped developing an antisense oligonucleotide-based approach to promote exon 2 inclusion and enhanced GAA enzyme activity to levels above the disease threshold (van der Wal, 2017). Preliminary *in vitro* data show promising rescue of acid alfa glucosidase activity in cells from Pompe patients by using this approach (Goina *et al*, 2017; van der Wal, 2017).

Pending Issues

The current understanding of lysosome biology and function is still evolving. There is a need for further characterization of these aspects that may provide critical information on the pathophysiology of lysosomal storage diseases.
New methodologies should be exploited to improve our knowledge on lysosomal biology and on lysosomal disease pathophysiology. These methodologies may also have a major impact of patient care, with more efficient diagnostic pathways and availability of biomarkers to follow disease progression and effects of therapies.
Current therapies for the treatment of lysosomal storage disease have significant limitations. Particularly, biodistribution in target organs, such as brain, is a critical issue as many of these disorders are associated with central nervous system involvement.
The understanding of disease pathophysiology is critical as it has the potential to identify novel therapeutic targets and to indicate new strategies for the treatment of these disorders.

Adjuvant therapies

In view of the emerging complexity of LSD pathophysiology, novel strategies are being explored that are based on an entirely different rationale and may represent adjunctive approaches to the treatment of LSDs. These approaches are not directed toward correction of the enzymatic defects and the causative gene mutations, or on modulation of the flux of substrates to lysosomes, but they are rather targeted to the manipulation of the pathways that are secondarily altered in LSDs.

For example, the abnormalities of the autophagic pathway are attractive targets. Indeed, manipulation of this pathway has been proposed as a therapeutic strategy for Pompe disease, providing some evidence of efficacy. *In vitro* and *in vivo* overexpression of TFEB also induced exocytosis, enhanced glycogen clearance, and resulted in some improvements in physical performance of Pompe disease mice (Spampanato *et al*, 2013; Gatto *et al*, 2017). *In vitro* overexpression of TFE3 triggered lysosomal exocytosis and resulted in efficient cellular clearance (Martina *et al*, 2014). This approach that has been explored so far through overexpression of master genes controlling the autophagic pathway may be difficult to translate into clinical applications. However, based on these proof-of-concept studies, alternative strategies based on the search for small-molecule drugs modulating autophagy may be envisaged, with a better potential for clinical translation.

Aberrant activation inflammation is another potential therapeutic target. For example, as neuroinflammation has been documented in several LSDs, the pathways that are involved in the activation of the inflammasome are now being considered as additional potential therapeutic targets. Pentosan polysulfate, a mixture of semisynthetic sulfated polyanions, has been shown to have anti-inflammatory effects in some LSDs, in particular targeting the activation TLR4 and the consequent secretion of proinflammatory cytokines and tumor necrosis factor (TNF)-α. This drug has been tested in mucopolysaccharidosis type I and II patients and in animal models of mucopolysaccharidosis types I, IIIA, and VI (Simonaro *et al*, 2010; Simonaro *et al*, 2016; Orii *et al*, 2019), and in *in vitro* models of Fabry and Gaucher disease (Crivaro *et al*, 2019). Intraperitoneal high-dose aspirin reduced neuroinflammation in mucopolysaccharidosis type IIIB mice, with significantly reduced transcript levels of MIP-1α, IL-1β, and GFAP (Arfi *et al*, 2011). A combination of Miglustat as a substrate-reducing agent, the Ca^{2+}-modulator curcumin, and a non-steroidal anti-inflammatory drug to target neuroinflammation was evaluated in Niemann-Pick disease type C1 mice and resulted into maintained body weight and motor function, reduced microglial activation, and delayed onset of Purkinje cell loss (Williams *et al*, 2014).

Other experimental therapeutic approaches have been directed toward correction of intralysosomal calcium levels in Niemann-Pick disease type C1 (Lloyd-Evans *et al*, 2008) and reduction in oxidative stress in Krabbe disease (Hawkins-Salsbury *et al*, 2012), while stimulation of the cytoprotective effect of HSP70 with the small-molecule arimoclomol in Niemann-Pick disease type C1 is under clinical evaluation (Kirkegaard *et al*, 2016).

Although these approaches directed toward correction of the secondary abnormalities in LSDs are not expected to be curative, they may be of help in improving quality of life and slow disease progression. It is possible to speculate that correction of these abnormalities may synergize with existing therapies. For example, in Pompe disease the block of autophagy has been shown to impact on the lysosomal trafficking of the recombinant enzyme used for ERT (Fukuda *et al*, 2006b). It may be conceivable that improving the status of autophagic pathways may translate into improved lysosomal delivery of the therapeutic enzyme.

It is possible that other potential therapeutic targets will be identified thanks to the precise characterization of the pathogenetic cascade of LSDs and will open new avenues to the treatment of LSDs.

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Conflict of interest

A. Ballabio is co-founder of CASMA Therapeutics and of Next Generation Diagnostics (NGD).

For more information

- NORD—National Association for rare diseases: https://rarediseases.org/rare-diseases/lysosomal-storage-disorders/
- Lysosomal Disease Network Patients & Families: https://lysosomaldiseasenetwork.org/
- National Gaucher Foundation: https://www.gaucherdisease.org/
- National Fabry Disease Foundation: https://www.fabrydisease.org/
- Sanfilippo Children Association: https://www.sanfilippo.org.au/
- Acid Maltase Deficiency Association: https://amda-pompe.org/
- European Reference Network for Hereditary Metabolic Disorders: https://metab.ern-net.eu/
- Tigem—The Telethon Institute of Genetics and Medicine: https://www.tigem.it/
- Telethon Foundation: https://www.telethon.it/

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